PATENT
ATTORNEY DOCKET 28385/35415

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
BEFORE THE BOARD OF PATENT APPEALS AND INTERFERENCES

Application of:	)	For: Anti-Viral Uses of Leflunomide Products
Williams *et al.*	)	
Serial No: 09/529,053	)	Group Art Unit: 1617
Filed: April 6, 2000	)	Examiner: S. Wang

APPELLANTS' BRIEF UNDER 37 C.F.R. §1.192

This brief is filed pursuant to 37 C.F.R. § 41.37 in support of the Notice of Appeal filed in this case on October 15, 2007. The Commissioner is authorized to charge any requisite fees under 37 C.F.R. § 41.20(b), and any other fees that should be submitted herewith, including fees for a petition for extension of time if necessary, to Deposit Account No. 13-2855 under reference no. 28385/35415.

This brief contains the following sections, including all of the items as required by 37 C.F.R. § 41.37 and M.P.E.P. § 1205.2:

I. REAL PARTY IN INTEREST

The real party in interest is James Williams, M.D., by virtue of (1) an assignment from his co-inventor Anita Chong, recorded December 6, 2007 at Reel 020204, Frame No. 0060, and (2) an assignment from his co-inventor W. James Waldman, Ph.D. to Ohio State University Research Foundation, recorded April 6, 2000 at Reel 10799, Frame No. 0010 and a subsequent assignment from Ohio State University Research Foundation to James Williams, recorded December 13, 2007 at Reel 020238, Frame No. 0836 (pursuant to the memorandum recorded November 30, 2005 at Reel 17080, Frame No. 0969).

II. RELATED APPEALS AND INTERFERENCES

There are no pending appeals or interferences related to the present Application.

III. STATUS OF CLAIMS

Claims canceled:	claims 1-33 and 43-44
Claims pending:	claims 34-42, 45 and 46
Claims withdrawn:	none
Claims rejected:	claims 34-42, 45 and 46
Claims on appeal:	claims 34-42, 45 and 46

IV. STATUS OF AMENDMENTS

The last amendment to the claims was an after-final amendment filed December 7, 2006, which was entered upon Appellants' Request for Continued Examination filed May 2, 2007. There are no other outstanding amendments.

V. SUMMARY OF CLAIMED SUBJECT MATTER

The present invention relates to treating viral infection by administering: (a) leflunomide product and (b) a pyrimidine compound that increases levels of the naturally occurring pyrimidine nucleotides (uridine, cytidine or thymidine). Leflunomide and related compounds were known in the prior art to have anti-inflammatory and immunosuppressive activity, and had been used to treat autoimmune disease and transplant rejection. No anti-viral activity had previously been reported for leflunomide or its metabolite. Appellants discovered and demonstrated in their application that leflunomide products exhibit anti-viral activity against a variety of viruses.

Applicants also discovered that co-administration of a pyrimidine compound that increases levels of uridine, cytidine or thymidine reduces the toxicity of leflunomide product while maintaining its anti-viral effectiveness.

As noted at page 1, lines 17-20, the active metabolite of leflunomide exhibits two mechanisms of action: inhibition of protein tyrosine kinase activity, and inhibition of dihydroorotate dehydrogenase, a key enzyme in the biosynthesis of pyrimidine nucleotide triphosphates. The latter activity of leflunomide, inhibition of pyrimidine nucleotide synthesis, leads to reduced pyrimidine nucleotide levels and resulting toxicity. However, as evidenced in the examples, Applicants discovered that inhibition of pyrimidine nucleotide synthesis was ***not*** necessary for anti-viral activity, and that restoring normal pyrimidine nucleotide levels with pyrimidine compounds did not interfere with the anti-viral activity of leflunomide product. In fact, utilizing the claimed methods safely permits a higher dose of leflunomide product to be administered, with correspondingly greater anti-viral effect.

Claim 34 is directed to methods of treating patients with viral infection by co-administration of (a) leflunomide product and (b) a pyrimidine compound in an amount effective to enhance serum levels of uridine, cytidine or thymidine. See page 20, lines 1-22.

Dependent claims 35-38 and 45 recite specific leflunomide products.

Dependent claims 39-40 recite specific sets of viruses.

Dependent claims 41 and 42 recite specific pyrimidine compounds.

Independent claim 46 is directed to methods of treating patients with viral infection by administering (a) a leflunomide product and (b) a pyrimidine compound without antiviral activity. See page 20, lines 1-22.

VI. ISSUES PRESENTED

(a) Did the Examiner commit legal error by (i) requiring literal written description for the claims in the specification, and (ii) failing to give due weight to declaratory evidence regarding what one of ordinary skill in the art would have understood from the specification?

(b) Did the Examiner commit legal error by (i) instituting an obviousness rejection despite a failure of the combined references to disclose all elements of the claim, and (ii) ignoring evidence of unexpected results?

VII. GROUNDS OF REJECTION TO BE REVIEWED ON APPEAL

Claim 46 stands rejected under 35 U.S.C. 112, first paragraph, as allegedly containing new matter because the phrase, "pyrimidine compound without antiviral activity" did not appear in the application as originally filed.

There are no art rejections with respect to claim 46.

Claims 34, 35, 40, 41 and 45 stand rejected under 35 U.S.C. 103 as allegedly obvious over Weithmann et al., U.S. Pat. No. 5,556,870 (hereafter "Weithmann"), in view of Hammer, *AIDS*, 10: supp. 3, s1-s11 (1996) (hereafter "Hammer") and Colacino, *Antiviral Res.* 29:125-39 (1996) (hereafter "Colacino").

Claims 39 stands rejected under 35 U.S.C. 103 as allegedly obvious over Weithmann in view of Hammer and Colacino, and further in view of Flamand et al., CAPLUS Abstract, AN 1991:581163 (hereafter "Flamand").

Claim 34-42 and 45 stand rejected under 35 U.S.C. 103 as allegedly obvious over Coghlan et al., Int'l Publication No. WO 94/24095 (hereafter "Coghlan") in view of McChesney et al., *Transplantation*, 57(12):1717-1722 (1994) (hereafter "McChesney") and further in view of Hammer and Colacino.

There are no rejections under 35 U.S.C. 112, first paragraph, applied to claims 34-42 and 45.

VIII. ARGUMENT

A. The Rejection Under 35 U.S.C. 112, First Paragraph

1. *Claim 46*

Claim 46 is the only claim that stands rejected under 35 U.S.C. 112, first paragraph. The basis asserted for the rejection is that the phrase "pyrimidine compound without antiviral activity" lacks written descriptive support and is new matter. In response to the written description rejection, Appellants submitted a Declaration of Walter Atwood, Ph.D. on December 21, 2006 (hereafter "Atwood Declaration") (Appendix B page 2).

The Examiner erred by (a) requiring explicit and literal support for this phrase, contrary to applicable case law, and (b) failing to consider or accord due weight to factual declaratory evidence that one of ordinary skill in the art would understand from reading the specification that "pyrimidine compound" as described in the specification would be understood to be without antiviral activity.

The Examiner states that the limitation "a pyrimidine compound without antiviral activity" lacks written descriptive support, because the "application does not particularly

exclude antiviral pyrimidine compounds."[1] However, such a requirement for explicit literal support has no basis in law. Rather, it is sufficient if the originally-filed application conveyed to one having ordinary skill in the art that the applicant had possession of the concept of what is claimed. *See, e.g., Ex parte Parks*, 30 U.S.P.Q.2d 1234, 1236 (B.P.A.I. 1994); *In re Alton*, 76 F.3d 1168, 1172 (Fed. Cir. 1996). Where an element is not literally or explicitly described, it may nonetheless be inherent in the specification if one of ordinary skill in the art, reading the original disclosure, can reasonably discern the limitation at issue. *Crown Operations Int'l, Ltd. v. Solutia, Inc.*, 289 F.3d 1367, 1376 (Fed. Cir. 2002).

In *Parks*, a negative claim limitation was found to be inherently part of the specification, even though there was no literal support for it, because an expert declaration stated that one having ordinary skill in the art would have recognized that the negative limitation was necessary for the disclosed reaction. *Parks*, 30 U.S.P.Q.2d at 1236-1237. The claim at issue recited the decomposition of nitrogen to nitric oxide "in the absence of a catalyst." *Id.* at 1235. The descriptions of the reaction never recite a catalyst, nor is a catalyst explicitly excluded from the reaction. However, an expert stated in his declaration that one having ordinary skill in the art would have recognized that the reaction decomposing nitrogen into nitric oxide detailed in the specification is conducted without a catalyst. *Id.* at 1236. Based on the facts stated in the expert declaration, the Board found that the originally-filed disclosure did convey to one having ordinary skill in the art the concept of effecting the reaction in the absence of a catalyst, and that there was written description for the claim. *Id.* at 1237.

The scenario in *Parks* is analogous to the situation at hand because the concept of using pyrimidine compounds "without antiviral activity" is conveyed by the specification's disclosure despite the absence of an explicit statement of this phrase. As in *Parks*, Appellants have supplied an expert declaration with factual evidence and reasoning why one of ordinary skill in the art would have understood that the inventors were claiming the administration of pyrimidine compounds without antiviral activity.

For example, paragraph 7 of the Atwood Declaration [Appendix B page 2] explains that the ordinary skilled artisan would have understood the statements in the application at

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[1] Page 2, paragraph 4 and page 7, paragraph 13 of the July 26, 2007 Action.

page 14, lines 1-6, reproduced below, as contemplating administration of a pyrimidine compound to reduce the toxicity of the leflunomide product, ***not for any anti-viral effect***:

> According to another aspect of the invention, a leflunomide product is co-administered with a pyrimidine, such as uridine, in order to reduce its toxicity while maintaining its therapeutic effectiveness. It is contemplated that co-administration with a pyrimidine may allow administration of an anti-viral therapeutically effective amount of leflunomide product with reduced immunosuppressive or toxic side effects.

Moreover, for the reasons explained in paragraph 8 of the Atwood Declaration, "pyrimidine compounds" as that term is defined in the specification ***would inherently be "without antiviral activity"*** because they supply naturally occurring pyrimidine nucleotides, cytidine, thymidine and/or uridine. Page 20, lines 12-14 states that "pyrimidine compound" is defined as "compounds useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides (uridine, cytidine and thymidine)." Dr. Atwood stated that uridine, cytidine and thymidine are naturally occurring and have no anti-viral effect. Dr. Atwood further stated that compounds that supply these nucleotides would not be expected to have an anti-viral effect. Paragraph 9 of the Atwood Declaration discussed data in the specification confirming that uridine lacks anti-viral effect.

Finally, Dr. Atwood stated at paragraph 10 that "It is clear, from reading the application, that the pyrimidine compounds to be co-administered with leflunomide product were not intended to have antiviral activity. I base my conclusion on the facts that (a) the stated purpose of the pyrimidine compound was to reduce toxicity of the leflunomide product, not for an anti-viral effect, and (b) the definition of pyrimidine compound [as a compound that increases naturally occurring nucleotide levels] excludes pyrimidine compounds with anti-viral activity."

In this case, the Examiner failed to consider or address any of the specific factual statements in the Atwood Declaration that formed the basis for his expert opinion in paragraphs 10 and 11. There is no discussion of the contents of the Atwood Declaration at page 7, paragraph 13 of the July 26, 2007 office action, merely a brief statement that "the arguments as to the scope of 'pyrimidine compounds' are not persuasive" and repeated statements that the application "does not exclude" anti-viral pyrimidine compounds. Moreover, any statements by the Examiner in rebuttal to the facts set forth in the Atwood declaration should have been supported by objective evidence in the form of an art-accepted reference, not simply by the Examiner's own opinions. *See In re Spormann*, 363 F.2d 444,

447, 150 U.S.P.Q. 449, 452 (C.C.P.A. 1966) ("if the Patent Office wishes to rely on [specific knowledge in the prior art], it must produce some reference showing what such knowledge consists of"); *In re Ahlert*, 424 F.2d 1088, 1091, 165 U.S.P.Q. 418, 420-21 (CCPA 1970).

The Federal Circuit has held that a summary dismissal of an expert's declaration, without an adequate explanation of why the declaration failed to rebut the prima facie case of inadequate description, is erroneous. *Alton*, 76 F.3d at 1174. As in *Alton*, the thrust of the Examiner's response to the Atwood declaration was that the specification must explicitly describe the claimed terms, and the Examiner ignored the facts set forth in the declaration as to what one of ordinary skill in the art would have understood the specification to describe. *See Alton*, 76 F.3d at 1176. The Examiner's summary dismissal here of the Atwood Declaration, as in *Alton*, was erroneous.

Thus, the Examiner erred by dismissing the Atwood Declaration and by requiring explicit support for the negative limitation "without antiviral activity". For all of the above reasons, Appellants respectfully request that the Board reverse the Examiner.

B. The Rejection Under 35 U.S.C. 103

The Examiner erred in instituting an obviousness rejection because (1) the cited references fail to teach or suggest all elements of the claims, and (2) the Examiner ignored evidence of unexpected results associated with the claimed treatment methods.

1. References cited relating to leflunomide products

Weithmann neither discloses nor suggests that leflunomide product has anti-viral activity. Weithmann was cited for teaching a method of using leflunomide to treat a disorder in which interleukin (IL)-1 beta levels are elevated. Weithmann claims use of leflunomide in a wide-ranging list of disorders, including leukemia, hepatitis, increased cartilage absorption, HIV infection, Alzheimer's disease, muscle breakdown, meningitis, microbacterial infections, thromboses, arteriosclerotic depositions, or elevated fat level and joint destruction. The Examiner acknowledges at page 3, paragraph 7 of the July 26, 2007 office action that "Weithmann et al. do not teach expressly the employment of addition [sic] pyrimidine antiviral agent in the method."

Flamand was cited as a secondary reference that assertedly teaches that herpesvirus infection is associated with elevated interleukin 1 beta levels.

Coghlan was cited for assertedly teaching general structures that encompass leflunomide products that also fall within the formulas recited in claims 38 and 44. The background at pages 3-4 states that it is an object of the invention to provide methods of treating a long list of unrelated diseases that is nearly two pages long. Based on this list, the Examiner asserted that the compounds disclosed are useful for treating hepatitis and CMV infection, although the summary only discloses methods of producing immunosuppression and the application is entitled "Immunosuppressive Agents." The Examiner admits that "Coghlan et al. does not teach expressly the employment [of] leflunomide or its metabolite, or the particular amount herein for treating viral infections." The Examiner further acknowledged at page 5, paragraph 10 of the July 26, 2007 office action that "Coghlan et al. (WO 94/24095) do not teach expressly the employment of pyrimidine compound in the method."

McChesney was cited (e.g., abstract) for assertedly teaching that leflunomide and its metabolite A771726 are effective in preventing viral infection. The Examiner appears to have misunderstood the statements in McChesney, however, which merely indicate that the immunosuppressive effects of leflunomide did not result in an increased incidence of bacterial or viral infection. For example, the abstract states that "Leflunomide is an isoxazole with newly discovered immunosuppressive properties. . . . .we subjected leflunomide to the rigorous canine renal transplantation model in a dose response protocol. . . . Even at a high dose of 16 mg/kg/day, no viral or bacterial infections were noted." This statement cannot be construed as a teaching that leflunomide has anti-viral activity.

2. References cited relating to pyrimidine compounds

Hammer was cited for assertedly teaching that "several pyrimidine compounds, including uridine compounds, are known antiviral agents." However, Hammer discloses no naturally occurring pyrimidine nucleosides or nucleotides. Instead it discloses non-naturally occurring ***nucleoside analogs*** that act to inhibit an enzyme responsible for DNA synthesis, reverse transcriptase.

Colacino was cited for assertedly teaching that fialuridine (FIAU), another non-natural ***nucleoside analog***, is useful for treatment of hepatitis and herpes virus infections.

3. The rejection of claims 34, 35, 40, 41 and 45 as obvious over Weithmann in view of Hammer and Colacino

The Examiner erred in rejecting claims 34, 35, 40, 41 and 45 as obvious over Weithmann in view of Hammer and Colacino because (1) the cited references fail to teach or suggest all elements of the claims, and (2) the Examiner ignored evidence of unexpected results associated with the claimed treatment methods.

a. Failure to disclose or suggest all elements

(i) Failure to disclose or suggest administration of a pyrimidine compound that increases levels of uridine, cytidine or thymidine.

It is an axiomatic principle that all claim limitations must be taught or suggested by the prior art to establish obviousness of a claimed invention. M.P.E.P. §2143.03 (2007) (citing *In re Royka*, 490 F.2d 981, 180 USPQ 580 (CCPA 1974)). Here, the element missing from all of the cited reference disclosures is the administration of a pyrimidine compound that increases "levels of uridine, cytidine or thymidine."

The Examiner acknowledged that the primary reference Weithmann, which relates to leflunomide, does not teach employment of an additional pyrimidine compound in the treatment method. To supply the missing element, the Examiner cited Hammer and Colacino for motivating administration of pyrimidine compounds to treat viral infection. However, *neither Hammer nor Colacino discloses pyrimidine compounds that "enhance serum levels of uridine, cytidine or thymidine"* as recited in claim 34.

Hammer and Colacino both disclose nucleoside analogs, which do ***not*** enhance serum levels of the naturally occurring nucleotides uridine, cytidine or thymidine. The nucleoside analogs discussed in Hammer and Colacino exert their anti-viral effect precisely because they are non-natural analogs. In other words, the basis for their anti-viral activity is their ability to act *unlike* natural nucleosides or nucleotides. For example, the nucleoside analogs of Hammer act by inhibiting the activity of reverse transcriptase, an enzyme involved in viral DNA synthesis.

Hammer and Colacino actually teach away from treating viral infection with a pyrimidine compound that supplies naturally occurring nucleotides such as uridine, cytidine

or thymidine, because such compounds would not have anti-viral activity. Evidence supporting this position is found in paragraph 8 of the Atwood Declaration, which states that pyrimidine compounds which do supply uridine cytidine, or thymidine *would not be expected to have an anti-viral effect.*

Conversely, administering the anti-viral nucleoside analogs of Hammer or Colacino would not provide the unexpected benefits of the claimed methods (reduction in leflunomide product toxicity). The reduction in toxicity with the claimed methods occurs because the supply of naturally occurring nucleotides is increased, an effect that would not be expected from administering non-natural nucleoside analogs. Supporting evidence for this fact is found in Sommadossi et al., *Antimicrob. Agents Chemother.* 32(7): 997-1001 (1988) [Appendix B page 14] and Walker et al., *Antivir. Ther.* 10 suppl. 2:M117-23 (2005) (abstract) [Appendix B page 19], showing that the nucleoside analogs of Hammer themselves cause toxic effects resulting from their interference with normal DNA synthesis.

The Examiner's statement at page 8, paragraph 13 of the July 26, 2007 office action that "The antiviral agents cited on the record are deemed to meet such limitation [of the claims]", *i.e.,* that the non-natural nucleoside analogs of Hammer and Colacino would increase serum levels of uridine, cytidine or thymidine, is unsupported by any factual evidence. An examiner's assertions of technical facts, particularly here where Applicants have submitted directly contradictory factual evidence, should be supported by objective evidence such as citation to some reference work recognized as a standard in the pertinent art. *See In re Ahlert*, 424 F.3d 1088, 1091 (C.C.P.A. 1970).

There is no express teaching in Hammer and Colacino to administer an amount of pyrimidine compound effective to enhance serum levels of uridine, cytidine or thymidine, as recited in claim 34. The Examiner's position appears to be that this element is inherent in administration of the nucleoside analogs. See, e.g., the statement at page 8, paragraph 15 of the July 26, 2007 office action that "applicant has recognized another advantage which would flow naturally from following the suggestion of the prior art." However, when relying on a theory of inherency, "the Examiner must provide a basis in fact and/or technical reasoning to reasonably support the determination that the allegedly inherent characteristic necessarily flows from the teachings of the applied prior art." *Ex parte Levy*, 17 USPQ2d 1461, 1464 (B.P.A.I. 1990); *see also In re Robertson*, 169 F.3d 743, 745, 49 USPQ2d 1949, 1950-51 (Fed. Cir. 1999). In this case, the Examiner's statement is unsupported by any factual

evidence that the recited property is "necessarily present" in the pyrimidine compounds of the cited art. Appellants' evidence supports the opposite conclusion, i.e., that the reduction in leflunomide product toxicity would ***not*** flow naturally from administration of the nucleoside analogs of Hammer and Colacino.

Thus, the obviousness rejection was improper and should be reversed because the cited references do not teach all elements of the claims, e.g., administration of a pyrimidine compound that increases "levels of uridine, cytidine or thymidine."

(ii) Failure to disclose or suggest administration of an amount of leflunomide product "therapeutically effective" for viral infection

None of the references discloses or suggests administration of an amount of leflunomide product that is therapeutically effective for viral infection. At the most, Weithmann might be argued to teach administration of an amount that reduces IL-1 beta levels. Hammer and Colacino have no disclosure relevant to dosing of leflunomide product.

For this reason also, the obviousness rejection was improper and should be reversed because the cited references do not teach all elements of the claims.

b. Evidence of unexpected results

Rebuttal evidence may include evidence of "secondary considerations" and evidence of unexpected results. *Graham v. John Deere Co.*, 383 U.S. 1, 17 (1966). For example, in the case of a claim to a combination, applicants may submit evidence or argument to demonstrate that (1) the elements in combination do not merely perform the function that each element performs separately; or (2) the results of the claimed combination were unexpected. Examination Guidelines for Determining Obviousness Under 35 U.S.C. 103 in View of the Supreme Court Decision in KSR Int'l Co. v. Teleflex, Inc., 72 Fed. Reg. 57,534 (Oct. 10, 2007). As noted in *KSR v. Teleflex* , "a court must ask whether the improvement is more than the predictable use of prior art elements according to their established functions." *KSR Int'l. Co. v. Teleflex, Inc.*, 550 U.S. ___, 127 S. Ct. 1727, 1740 (2007).

In the present claims, the elements in combination do not merely perform the function that each element performs separately. Even if one assumes, for the sake of argument, that the Examiner is correct in asserting that Hammer and Colacino motivate the treatment of viral

infection with any compound that includes a general 6-member pyrimidine ring moiety, none of the cited art would predict that pyrimidine compounds could relieve the toxicity associated with leflunomide products. Thus, the pyrimidine compound element, when combined with the leflunomide product element, is performing a different function than its allegedly established function of treating viral infection.

In addition, the beneficial results of the claimed combination, reduction of leflunomide product toxicity, were unexpected and could not have been predicted from the cited art. Moreover, this reduction in toxicity is accomplished without interfering with anti-viral activity. Evidence supporting this activity *in vitro* is found in the application as filed. Example 2 of the specification at pages 26-27 shows that co-treatment of virally infected cells with A771726 (a leflunomide product) and uridine did not interfere with the anti-viral activity of A771726 because infectious virus production was not significantly increased in the presence of uridine. These data also show that the pyrimidine did not improve anti-viral activity. Example 2 concludes that inhibition of viral activity by leflunomide product A771726 is independent of the inhibitory effects of this agent upon pyrimidine nucleotide synthesis.

Later-published evidence confirming these unexpected results *in vivo* is found, e.g., in Chong et al., Transplantation, 1999 Jul 15;68(1):100-9 [Appendix B page 4], which states that co-administration of uridine significantly reduced the toxicities associated with high-dose leflunomide, such as anemia, diarrhea and pathological changes in the small bowel and liver. The abstract states:

> Toxicities associated with high-dose leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. *These toxicities were significantly reduced by uridine co-administration.* [Emphasis added.]

WO 2006/014827 [Appendix B page 26] shows that another pyrimidine compound, orotic acid, also reduces the toxic side effects of leflunomide *in vivo*. Examples 2 and 3 show that co-administration of orotic acid reduced anemia and increased weight gain.

The unexpected benefit arises from the restoration of normal levels of naturally occurring pyrimidine nucleotides, and thus is commensurate in scope with the claim, which recites administration of a pyrimidine compound that increases levels of uridine, cytidine or thymidine (which are the naturally occurring pyrimidine nucleotides that are used as "building blocks" for DNA or RNA, see paragraph 8 of Atwood Declaration).

Thus, the obviousness rejection was improper and should be reversed because of Applicants' demonstrated evidence of a property, alleviation of leflunomide toxicity, entirely different from the antiviral effect purportedly disclosed in the prior art for pyrimidine compounds. The invention represents an improvement that is "more than the predictable use of prior art elements according to their established functions." *KSR*,127 S. Ct. at 1740.

2. The rejection of claim 39 as obvious over Weithmann in view of Hammer and Colacino, and further in view of Flamand

The Examiner erred in rejecting claim 39 under 35 U.S.C. 103 as obvious over Weithmann in view of Hammer and Colacino, and further in view of Flamand because (1) the cited references fail to teach or suggest all elements of the claim, and (2) the Examiner ignored evidence of unexpected results associated with the claimed treatment methods.

As noted above, Weithmann was cited for assertedly teaching use of leflunomide to treat disorders associated with elevated IL-1 beta. Claim 39 is directed to treatment of herpes virus infections, which were not recited among Weithmann's list of diverse disorders assertedly related to IL-1 beta. To remedy this deficiency, the Examiner cited Flamand as purportedly teaching that herpes virus infection is associated with elevated IL-1 beta levels. However, neither Weithmann nor Flamand teaches administration of an amount of leflunomide product that is "therapeutically effective" for viral infection, as recited in claim 34 (from which claim 39 depends). Although the Examiner has taken the position that optimization of dosages is routine, it is not obvious to optimize a dosage to achieve an effect (inhibition of viral growth rather than effect on IL-1 beta levels) that is not known or suggested in the cited art.

Furthermore, for all of the reasons discussed above in section B.1.a.i., none of the references discloses *pyrimidine compounds that "enhance serum levels of uridine, cytidine or thymidine"* as recited in claim 34. There is no express teaching of this element in any of the references, nor is this limitation an inherent property of the nucleoside analogs disclosed in the cited art (e.g. Hammer or Colacino).

For all of the reasons discussed above in section B.1.a.ii., the pyrimidine compounds recited in the claim are performing a toxicity-reducing function that is entirely different from the anti-viral function assertedly disclosed by the cited art. Moreover, Applicants have demonstrated further unexpected results, that the pyrimidine compounds alleviate

leflunomide toxicity without affecting its therapeutic efficacy. These results thus represent an improvement that is "more than the predictable use of prior art elements according to their established functions." *KSR*, 127 S. Ct. at 1740.

3. The rejection of claims 34-42 and 45 as obvious over Coghlan in view of McChesney and further in view of Hammer and Colacino

The Examiner erred in rejecting claim 34-42 and 45 under 35 U.S.C. 103 as obvious over Coghlan in view of McChesney and further in view of Hammer and Colacino because (1) the cited references fail to teach or suggest any elements of the claim, and (2) the Examiner ignored evidence of unexpected results associated with the claimed treatment methods.

Coghlan was cited for teaching general structures that encompass compounds that fall within the chemical formulas of claims 38 and 44, and that are assertedly useful for treating hepatitis and CMV. However, no anti-viral effect is demonstrated in Coghlan.

McChesney was cited (e.g., abstract) for assertedly teaching that leflunomide and its metabolite A771726 are effective in preventing viral infection. As noted above in section B.1., the Examiner appears to have misunderstood the statements in McChesney, which merely indicate that the immunosuppressive effect of high dose leflunomide, which increases susceptibility to infection, did not result in any observed bacterial or viral infections.

For all of the reasons discussed above in section B.1.a.i., none of the references discloses *pyrimidine compounds that "enhance serum levels of uridine, cytidine or thymidine"* as recited in claim 34. There is no express teaching of this element in any of the references, nor is this limitation an inherent property of the nucleoside analogs disclosed in the cited art.

For all of the reasons discussed above in section B.1.a.ii., the pyrimidine compounds recited in the claim are performing a toxicity-reducing function that is unexpected and entirely different from the anti-viral function assertedly disclosed by the cited art. Moreover, Applicants have demonstrated further unexpected results, that the pyrimidine compounds alleviate leflunomide toxicity without affecting its therapeutic efficacy. These results thus represent an improvement that is "more than the predictable use of prior art elements according to their established functions." *KSR*, 127 S. Ct. at 1740.

Thus, the obviousness rejection should be reversed because of Applicants' demonstrated evidence of an unexpected benefit of the claimed methods, alleviation of leflunomide toxicity, that is an entirely different property from the antiviral effect purportedly disclosed in the prior art for pyrimidine compounds.

C. Conclusion

For all of the reasons above in section A, the written description rejection applied to claim 46 should be reversed because the specification reasonably conveyed to the ordinary skilled artisan that the pyrimidine compounds administered, which are defined as supplying the naturally occurring pyrimidines uridine, cytidine and thymidine, were without antiviral activity. The Examiner's failure to consider Appellants' evidence relevant to this matter is also reversible error.

For all of the reasons above in section B, the obviousness rejection applied to claims 34-42 and 45 should be reversed because a proper case of obviousness is not established when all elements of the claims are not disclosed or suggested, and in addition because unexpected results commensurate in scope with the claim show nonobviousness of the claimed subject matter.

The Examiner's legal and factual errors thus necessitate reversal of all rejections and return of this case to the Examiner for appropriate allowance of the claims.

Respectfully submitted,

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APPENDIX A

LISTING OF CLAIMS

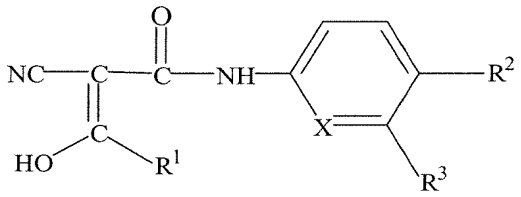
34. A method of treating a patient suffering from a viral infection comprising administering to said patient a therapeutically effective amount of a leflunomide product and administering to said patient a pyrimidine compound in an amount effective to enhance serum levels of uridine, cytidine or thymidine.

35. The method of claim 34 wherein the leflunomide product is N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide (HWA 486).

36. The method of claim 34 wherein the leflunomide product is N-(4-trifluoromethylphenyl)-2-cyano-3-hydroxycrotonamide (A771726).

37. The method of claim 34 wherein the leflunomide product is an amide of a malononitrile.

38. The method of claim 34 or 41 wherein the leflunomide product is a compound of formula:



(II)

wherein

R^1 denotes
a) methyl,
b) (C_3-C_6)-cycloalkyl,
c) (C_2-C_6)-alkyl, having at least 1 triple or double bond between the carbon atoms,

R^2 denotes
a) —CF_3 or
b) —CN,

R^3 denotes
a) (C_1-C_4)-alkyl or
b) hydrogen atom,

X denotes
a) —CH—group or
b) nitrogen atom,

the compound being present as such or in the form of a physiologically tolerable salt.

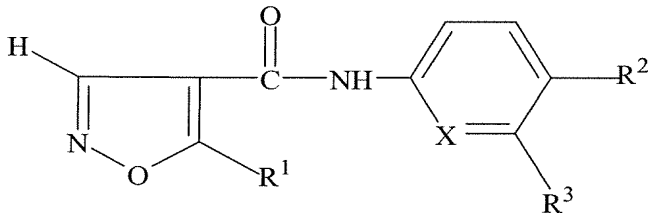
39. The method of claim 34, 35, 36 or 37 wherein the virus is a herpesvirus.

40. The method of claim 34, 35, 36 or 37 wherein the virus is selected from the group consisting of paramyxoviruses, picornaviruses, hepatitis viruses, CMV, HSV, measles virus, rhinoviruses, hepatitis B and hepatitis C.

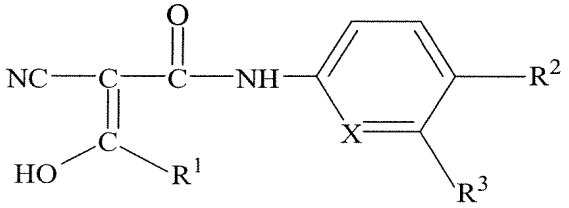
41. The method of claim 34 wherein the pyrimidine is uridine, orotic acid or orotidine.

42. The method of claim 37 wherein the pyrimidine is uridine, orotic acid or orotidine.

45. The method of claim 34 or 41 wherein the leflunomide product is a compound of formula I or II:



(I)



(II)

wherein in formula I or II

R^1 denotes
a) methyl,
b) (C_3-C_6)-cycloalkyl,
c) (C_2-C_6)-alkyl, having at least 1 triple or double bond between the carbon atoms,

R^2 denotes
a) —CF_3 or
b) —CN,

R^3 denotes
a) (C_1-C_4)-alkyl or
b) hydrogen atom, and

X denotes
a) —CH—group or
b) nitrogen atom;

the compound being present as such or in the form of a physiologically tolerable salt.

46. A method of treating a patient suffering from a viral infection comprising administering to said patient (a) a therapeutically effective amount of a leflunomide product and (b) a pyrimidine compound without antiviral activity.

APPENDIX B

EVIDENCE

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IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants: Williams *et al.*	)	
Serial No. 09/529,053	)	Group Art Unit: 1617
Filed: April 6, 2000	)	Examiner: S. Wang
For: ANTI-VIRAL USES OF LEFLUNOMIDE PRODUCTS	)	

DECLARATION OF WALTER ATWOOD, Ph.D.

Commissioner for Patents
P.O. Box 1450
Alexandria, Virginia 22313-1450

Dear Sir:

I, Walter Atwood, Ph.D., hereby declare as follows:

1. I am currently a Professor of Medical Science at Brown University. I received a B.S. in Microbiology from the University of Massachusetts at Amherst in 1986 and a Ph.D. in Neurovirology from the University of Massachusetts at Amherst in 1991. I am the author or co-author of numerous peer-reviewed journal articles and book chapters in the field of virology.

2. I have reviewed the text of U.S. patent application no. 09/529,053, attached hereto as Exhibit 1.

3. I have reviewed a copy of claim 34 attached hereto as Exhibit 2, which relates to a method of treating viral infection by co-administering a leflunomide product and a pyrimidine compound without antiviral activity.

4. I understand that the Examiner has objected to the recitation of a pyrimidine compound "without antiviral activity" in claim 34 because he believes that the application does not describe the use of pyrimidine compounds without antiviral activity. I make these statements to address the Examiner's objection.

5. My experience and education permit me to be familiar with what one of ordinary skill in the art would have understood upon reading the application at its March 11, 1998 priority date.

6. The application describes the anti-viral effects of leflunomide product and further describes methods of treating viral infection with leflunomide product. The application also states at page 14, lines 1-6 that:

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> According to another aspect of the invention, a leflunomide product is co-administered with a pyrimidine, such as uridine, in order to reduce its toxicity while maintaining its therapeutic effectiveness. It is contemplated that co-administration with a pyrimidine may allow administration of an anti-viral therapeutically effective amount of leflunomide product with reduced immunosuppressive or toxic side effects.

7. One of ordinary skill in the art as of March 11, 1998 would have understood, from reading the language quoted in paragraph 6, that the inventor(s) contemplated administration of a pyrimidine compound ***to reduce the toxicity of the leflunomide product, not for any anti-viral effect.*** In other words, it is the leflunomide product, not the pyrimidine compound, that would have anti-viral activity.

8. The definition of pyrimidine compound confirms that the contemplated pyrimidine compounds ***would not have anti-viral activity.*** A pyrimidine compound is defined at page 20, lines 12-14 of the application as "compounds useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides (uridine, cytidine and thymidine)." Uridine, cytidine and thymidine are naturally occurring nucleosides, which are used as a "building block" for DNA or RNA, and which have no anti-viral effect. Thus, compounds that supply these nucleosides would not be expected to have an anti-viral effect.

9. The fact that uridine, an exemplary pyrimidine compound, has no anti-viral activity is confirmed in Example 2, Figure 2, which shows that uridine [Ur] alone has no effect on the production of infectious virus. In contrast, the leflunomide product A771726 alone [A77], or A771726 plus uridine [A77+Ur], inhibited infectious virus production.

10. It is clear, from reading the application, that the pyrimidine compounds to be co-administered with leflunomide product were not intended to have antiviral activity. I base my conclusion on the facts that (a) the stated purpose of the pyrimidine compound was to reduce toxicity of the leflunomide product, not for an anti-viral effect, and (b) the definition of pyrimidine compound excludes pyrimidine compounds with anti-viral activity.

11. Therefore, one of ordinary skill in the art as of March 11, 1998, upon reading the application, would have understood that the inventors were claiming the administration of pyrimidine compounds ***without antiviral activity.***

12. I further declare that all statements made herein of my own knowledge are true, that all statements made on information and belief are believed to be true, and that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both (18 U.S.C. § 1001), and may jeopardize the validity of the application or any patent issuing thereon.

12/19/06
Date

Dr. Walter Atwood

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TRANSPLANTATION

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IN VIVO ACTIVITY OF LEFLUNOMIDE

PHARMACOKINETIC ANALYSES AND MECHANISM OF IMMUNOSUPPRESSION

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Background. **Leflunomide is an experimental drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection. The two biochemical activities reported for the active metabolite of leflunomide, A77 1726, are inhibition of tyrosine phosphorylation and inhibition of dihydroorotate dehydrogenase, an enzyme necessary for *de novo* pyrimidine synthesis. These activities can be distinctly separated *in vitro* by the use of uridine, which reverses the anti-proliferative effects of A77 1726 caused by inhibition of *de novo* pyrimidine synthesis. We report the effect of uridine on the *in vivo* immunosuppressive activities of leflunomide.**

Methods. **We first quantified the serum levels of A77 1726, the active metabolite of leflunomide, after a single treatment of leflunomide (5, 15, and 35 mg/kg). Additionally, we quantified the levels of serum uridine and of nucleotide triphosphates in the liver, spleen, and lymph nodes of Lewis rats after the administration of a single dose of uridine (500 mg/kg; i.p.). Lewis rats heterotopically transplanted with brown Norway or Golden Syrian hamster hearts were treated for 50 or 75 days with leflunomide (5, 15, and 35 mg/kg/day; gavage) alone or in combination with uridine (500 mg/**

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kg/day; i.p.). Hematocrits were determined and the levels of alloreactive or xenoreactive immunoglobulin (Ig)M and IgG were determined by flow cytometric analysis. The allograft and xenografts, small bowel, liver, kidney, and spleen were subjected to pathological examination.

Results. **A linear relationship was observed between the serum A77 1726 concentrations in Lewis rats and the dose of leflunomide administered. Peak A77 1726 concentrations were 20.9, 71.8 and 129.3 mg/l (77.5, 266.1 and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively. The concentration of uridine in the serum of normal Lewis rats is 6.5 μM; after i.p. administration of 500 mg/kg uridine, the serum uridine concentrations peaked at 384.1 μM in 15–30 min. The rapid elimination of uridine was not reflected in the lymphoid compartments, and the pharmacokinetics of pyrimidine nucleotides in the spleen resembled that of A77 1726. This dose of uridine, when administered daily (500 mg/kg/day, i.p.), weakly antagonized the immunosuppressive activities of leflunomide (5, 15, and 35 mg/kg/day) in the allotransplantation model. In contrast, in the xenotransplantation model, the same concentration of uridine completely antagonized the immunosuppressive activities of low-dose leflunomide (15 mg/kg/day) and partially antagonized the immunosuppressive activities of high-dose leflunomide (35 mg/kg/day). Toxicities associated with high-dose leflunomide (35 mg/kg/day) were anemia, diarrhea, and pathological changes in the small bowel and liver. These toxicities were significantly reduced by uridine co-administration.**

Conclusion. **These studies reveal that the blood levels of A77 1726 in Lewis rats satisfy *in vitro* requirements for both inhibition of *de novo* pyrimidine synthesis and protein tyrosine kinase activity. Our data also illustrate that the *in vivo* mechanism of immunosuppression by leflunomide is complex and is affected by at least the following four factors: type and vigor of the immune response, availability of uridine for salvage by proliferating lymphocytes, species being investigated, and concentration of serum A77 1726.**

Leflunomide [N-(trifluoromethylphenyl)-methylisoxazol-4-carboxamide; HWA 486 or SU101] is an experimental immunosuppressive drug with demonstrated ability to prevent and reverse acute allograft and xenograft rejection (reviewed in (*1*)). Leflunomide is rapidly converted *in vivo* to the active metabolite, A77 1726. The two biochemical activities ascribed to A77 1726 are inhibition of protein tyrosine kinases and of dihyroorotate dehydrogenase (DHO-DHase*), a key enzyme in the *de novo* synthesis of pyrimidine nucleotides (*2–6*). The significantly lower IC_{50} required *in vitro* to inhibit DHO-DHase relative to tyrosine kinases has led many researchers in this field to suggest that the *in vivo* antiproliferative and immunosuppressive activities of leflunomide, and its active metabolite, A77 1726, result from the inhibition of the enzymatic activity of DHO-DHase (*1*).

Several lines of evidence suggest that this hypothesis may not be supported *in vivo*. First, the serum uridine in human and rodents (5–15 μM (*7, 8*)) could be converted to pyrimidine nucleotides by the salvage pathway, resulting in a normalization of intracellular pyrimidine nucleotide levels, despite inhibition of *de novo* pyrimidine synthesis. Second, when A77 1726 is used *in vitro* at concentrations >50 μM, the antiproliferative activity on T cells and B cells could no longer be completely reversed by the addition of exogenous uridine, suggesting a second mode of activity at these concentrations that is independent of pyrimidine depletion (*9, 10*). Third, patients with a genetic defect in *de novo* pyrimidine synthesis, hereditary orotic aciduria, do not have undue susceptibility to infection, indicating that they are not significantly immunosuppressed (reviewed in (*11*)). *In vitro* cellular immune defects have been reported in some of these patients; however, these immune defects can be attributed to lymphopenia arising from a generalized defect in hematopoiesis, rather than to an intrinsic inability of T cells to proliferate in response to antigen stimulation (*12, 13*).

These observations prompted us to examine whether the immunosuppressive activities of leflunomide in rats and mice are mediated by the inhibition of *de novo* pyrimidine synthesis. *In vitro* studies suggest that the effects of A77 1726 that are caused by inhibition of *de novo* pyrimidine synthesis can be reversed with uridine. Using a similar approach, we report the effects of uridine on the *in vivo* immunosuppressive activities and toxic side effects of leflunomide in mice and rats.

MATERIALS AND METHODS

Extraction and quantitation of A77 1726 from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of 5, 15, or 35 mg/kg/day leflunomide by gavage. Serum was collected at the indicated times and stored at 70°C before extraction. Serum, 50 μl, was mixed with 25 μl 4′-(trifluoromethyloxy)-acetanilide (TFMO; Sigma, St. Louis, MO), 200 μl 0.5 M HCl, and 4 ml extraction solvent (1:1 pentane and dichloromethane; Sigma). The mixture was vortexed for 1 hr, then centrifuged at 2000 g for 5 min (Centra-8, IEC, Needham Heights, MA). After freezing the mixture for 1 hr at −20°C, the organic phase was decanted into a new tube and dried under a hood at room temperature. Then 200 μl of reconstituting solution (1:1 acetonitrile and water) was added, and the mixture was vortexed and centrifuged at 2000 g for 2 min. Finally, 65 μl of the mixture was analyzed by high-performance liquid chromatography (HPLC) (Waters, Milford, MA), using a Symmetry C18 column (4.6 × 250 mm; Waters). A77 1726 was separated with a mobile phase composed of 50% acetonitrile and 50% buffer (25 mM KH_2PO_4). The corresponding peak of A77 1726 was compared with a standard of purified A77 1726 (a gift from Robert R. Bartlett, Hoechst Marion Roussel, Wiesbaden, Germany), and the concentrations were calculated on the basis of a standard curve of purified A77 1726.

Extraction and quantitation of uridine from serum. For single-dose pharmacokinetics, Lewis rats not receiving transplants were treated with a single dose of uridine. Serum was collected at the indicated times and stored at 70°C before extraction. Serum samples were diluted two fold in 0.9% NaCl, and uridine was extracted by the addition of an equal volume of 0.8 M trichloric acid and then neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon. Serum uridine was detected by HPLC, using a Lichrosorb-10RP-18 column (Whatman, Alltech, Deerfield, IL) and an elution solution (5 mM KH_2PO_4, pH 3.8), at a flow of 1 ml/min. The uridine peak was identified by its retention time and spectrum compared with a uridine standard (Sigma). Uridine concentrations were calculated on the basis of a standard curve.

Extraction and quantitation of nucleotide triphosphate from tissues. For single-dose pharmacokinetic analysis, normal Lewis rats were treated with a single dose of uridine. The spleen, lymph nodes,

* Abbreviations used: DHO-DHase, dihydroorotate dehydrogenase; Ig, immunoglobulin; HPLC, high-performance liquid chromatography; Ka, rate of absorption; Ke, rate of excretion; PCV, packed cell volume.

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and liver (100 mg tissue) were homogenized and nucleotide triphosphates were extracted with 0.4 M trichloric acid and neutralized with an equal volume of 0.5 M tri-n-octylamine in Freon 113, as previously described (*9*, *14*). Nucleotides were separated using a Whatman anion exchange column (Particil 10 SAX, Alltech) and a linear gradient elution of potassium phosphate buffer, pH 4.5 (10–500 mM). The corresponding peaks of four nucleotides were detected by HPLC (Waters), and the concentrations were calculated on the basis of a standard curve of purified nucleotides (Sigma).

Pharmacokinetic and statistical analysis. The pharmacokinetic analysis of serum A77 1726, uridine, and tissue UTP levels were conducted using a nonlinear regression analysis with a Gaussian algorithm. The time-concentration data were fitted to the open, one-compartment, extravascular model:

$$C_t = C^\circ(\exp(-K_e{*}t) - \exp(-K_a{*}t))$$

where C_t, C°, K_e, K_a, and t are the serum concentration at time t, the theoretical initial concentration, the excretion constant, the absorption constant, and the time after drug administration. The best fit values for C°, K_e, and K_a were used to calculate the terminal half-life ($T_{1/2}$) using the formula: $T_{1/2} = 0.693/K_e$. The area under the curve was calculated using the trapezoidal method. Statistical differences between pharmacokinetic parameters were analyzed using a *t* test or analysis of variance.

Transplantation model and drug treatment. Lewis or brown Norway rats, and Golden Syrian hamsters were purchased from Harlan Labs (Indianapolis, IN). Balb/c and C3H mice were purchased from Jackson Labs (Bar Harbour, ME). Heart grafts were heterotopically transplanted into the abdomen of the recipients after a modified protocol described by Ono *et al.* (*15*). Leflunomide (5–35 mg/kg/day, custom synthesized for research purposes) was suspended in 1% carboxymethyl cellulose and administered by gavage. Uridine (Sigma) was dissolved in 0.9% NaCl for daily i.p. injections. The transplanted hearts were monitored daily, and rejection was defined as the complete cessation of pulsations in the transplanted heart.

Packed cell volume. Rats were bled every 2 weeks or on the day they were killed, through the orbital vein, using a microhematocrit capillary tube (Baxter, Deerfield, IL). The blood was centrifuged for 15 mins at 550 g, and the percentage of packed cell volumes was determined with a micro-hematocrit capillary tube reader (Critocaps, Oxford Lab).

Quantification of allo-specific and hamster-specific IgM and IgG titers. Quantification of allo-specific or hamster-specific antibodies was performed, as previously described (*16*, *17*). Lymphocytes (5×10^5) from lymph nodes isolated from brown Norway rats or erythrocytes (10^6) from Golden Syrian hamster were incubated with diluted, heat-inactivated test serum or control naive Lewis rat serum (1:20 dilution) for 30 min at 4°C. Lymphocytes were washed with phosphate-buffered saline, and erythrocytes were washed in 4% (weight/volume) sodium citrate/phosphate-buffered saline. The cells were then stained with phycoerythrin-conjugated F(ab')2 anti-rat immunoglobulin (Ig)M or fluorescein isothiocyanate-conjugated F(ab')2 anti-rat IgG (Jackson ImmunoResearch, West Grove, PA). After staining, the erythrocytes and lymphocytes were washed, fixed in 1% formalin, and analyzed using a flow cytometer (Ortho Cytoron Absolute, Ortho Diagnostic Systems, Raritan, NJ).

Histology and immunohistochemistry. Sections of the spleen, liver, kidney, and small bowel were collected, imbedded in frozen tissue matrix CO.C.T. compound (Sakura Finetek U.S.A., Torrance, CA), and snap-frozen in liquid nitrogen. Sections of these tissues, 5 μm, were made and fixed in 10% formalin. These sections were then stained in hematoxylin and eosin solutions. Allografts were scored according to a modified cardiac biopsy grading by Billingham *et al.* (*18*). Grade 0 (no acute rejection) indicates no evidence of acute rejection or myocyte damage; Grade 1A (focal, mild acute rejection) indicates focal, perivascular, or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 1B (diffuse, mild, acute rejection) indicates a more diffuse, perivascular or interstitial infiltrate of mononuclear cells with no myocyte damage; Grade 2 (focal, moderate acute rejection) indicates a few focal aggressive inflammatory infiltrate with focal myocyte damage; Grade 3A indicates multifocal aggressive inflammatory infiltrate with myocyte damage; Grade 3B indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis; and Grade 4 indicates diffuse aggressive inflammatory infiltrate with myocyte necrosis, hemorrhage, edema, and vasculitis. Sections for immunohistochemical analysis were fixed in cold acetone and stained with monoclonal antibodies against rat IgM, IgG, TCR$\alpha\beta$, and ED1, using a modified ABC method, as previously described (*16*, *19*).

RESULTS

Single dose pharmacokinetics of A77 1726. Lewis rats received by gavage three different doses of leflunomide (5, 15, 35 mg/kg). Sera were harvested from 4–6 individual rats at the indicated times, and the active metabolite of leflunomide, A77 1726, in the serum was extracted and quantified by HPLC. Increasing concentrations of leflunomide resulted in a dose-dependent increase in A77 1726 in the serum (Fig. 1A). A linear relationship was observed between the peak concentrations and the area under the curve, and the dose of A77 1726 administered (Fig. 1B). The A77 1726 peak concentrations were 20.9, 71.8, and 129.3 mg/l (77.5, 266.1, and 478.8 μM) for the 5, 15, and 35 mg/kg doses of leflunomide, respectively (Fig. 1A). These peak concentrations were observed at 6–8 hr (Fig. 1A), and the mean terminal half-life ($T_{1/2}$) ranged from 3.5 to 5.0 hr, irrespective of the administered leflunomide dose (Table 1). The excretion constant (Ke) and absorption constant (Ka) were not statistically significant between treatment groups ($P > 0.05$), and the excretion of A77 1726 followed first-order kinetics even at highest dose (Table 1). Similar analyses performed with Balb/c mice given a single, oral dose of leflunomide (35 mg/kg) revealed similar peak A77 1726 concentrations (140 mg/l=514 μM) and Ka, compared with Lewis rats; however, the Ke, was significantly

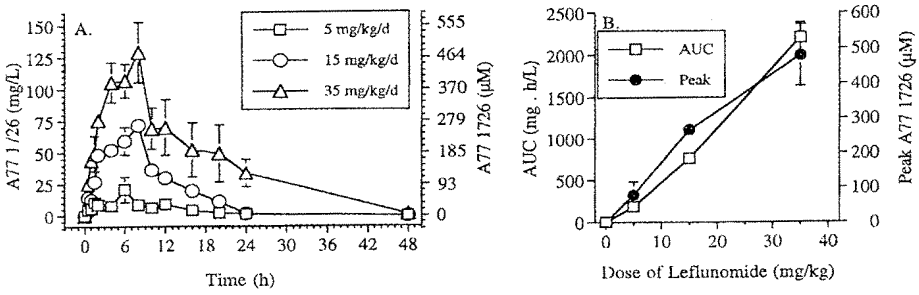

FIGURE 1. (A) Pharmacokinetics of A77 1726 after a single, oral dose of leflunomide in Lewis rats. Serum was harvested at the indicated times, and A77 1726 concentrations determined by HPLC. Data are presented as mean concentrations of 4–6 rats/group, and bars represent SEM. (B) The relationship between the AUC (mg · h/L) and peak concentrations of A77 1726 (μM) and the dose of leflunomide administered.

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TABLE 1. Pharmacokinetic parameters of serum A77 1726 following leflunomide administration

Treatment	Ke (ml · min/kg)	Ka (ml · min/kg)	T1/2 (h)	AUC (mg · h/L)
Single-dose leflunomide treatment in Lewis rats				
5 mg/kg leflunomide	0.258±0.112	0.484±0.138	4.42±0.16	183.0±19.6
15 mg/kg leflunomide	0.190±0.005	0.197±0.004	3.49±0.19	764.5±24.0
35 mg/kg leflunomide	0.150±0.023	0.204±0.046	4.97±0.77	2207.3±173.8
Single-dose leflunomide treatment in Balb/c mice				
35 mg/kg leflunomide	0.073±0.032	0.567±0.132	15.02±4.57	3446.5±973.3

slower ($P<0.05$; Table 1) resulting in a longer terminal half live of serum A77 1726.

Single dose uridine pharmacokinetics. *In vitro* and *in vivo* studies have indicated that uridine can be used to counter the effects resulting from the inhibition of *de novo* pyrimidine synthesis (*2–6, 14, 20*). We measured the levels of serum uridine in Lewis rats before and after the administration of a single dose of uridine (500 mg/kg, i.p.). Consistent with previous reports, the mean concentration of uridine in the serum of normal Lewis rats was 6.5±0.9 μM (n=13) (*7*). A single dose of uridine (500 mg/kg) administered i.p. resulted in a rapid increase in the concentrations of serum uridine. Maximum concentrations were observed within 15–30 min and reached a peak concentration of 384.1±53.5 μM. Serum uridine was rapidly cleared and returned to baseline 4 hr after uridine administration (Fig. 2A).

Intracellular nucleotides following a single dose of uridine. We next determined whether the elevated concentrations of serum uridine resulted in increased intracellular pyrimidine nucleotides in the spleen, lymph nodes, and liver. A cohort of 26 Lewis rats were treated with a single dose of uridine (500 mg/kg, i.p.). The rats were killed after 0, 1, 3, 6, 12, and 24 hr (n=4–5 per group), and lymph nodes and approximately 100 mg of liver and spleen tissue harvested. The nucleotides were extracted from the tissues by trichloroacetic acid, and the concentrations of tissue UTP, CTP, ATP and GTP determined by HPLC. The administration of uridine resulted in 4.1-, 3.6-, 2.4-, and 1.5-fold increases in UTP, CTP, ATP, and GTP, respectively, in the spleen (Fig. 2B–D). The tissue nucleotide concentrations remained at these levels for up to 6 hr and gradually declined to baseline 24 hr after uridine administration. The $T_{1/2}$ of tissue UTP in the spleen was 12.72 hr, with a Ka and Ke of 0.216 and 0.05 ml · min/kg, respectively. Statistical analysis indicated that the pharmacokinetics of UTP levels in the spleen after uridine administration was not significantly different ($P>0.05$) from the pharmacokinetics of A77 1726 after the administration of leflunomide.

After uridine administration, the levels of UTP and CTP increased 4.9- and 2.8-fold, respectively, in the liver tissue and 1.7- and 1.3-fold, respectively, in lymph node cells (Fig. 2B–D). The levels of ATP and GTP were also elevated 3.1- and 2.9-fold, respectively, in the liver and 1.5- and 1.3-fold in the lymph node cells after uridine administration (data not shown). We do not have an explanation for the concomitant increase in ATP and CTP levels but speculate that it could reflect uridine-stimulated increases in metabolic activity in these tissues.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in Lewis rats. Brown Norway hearts transplanted into untreated Lewis rats were rejected in 6–8 days. Treatment with 5 or 15 mg/kg/day of leflunomide resulted in the survival of the allografts for >50 days, whereas

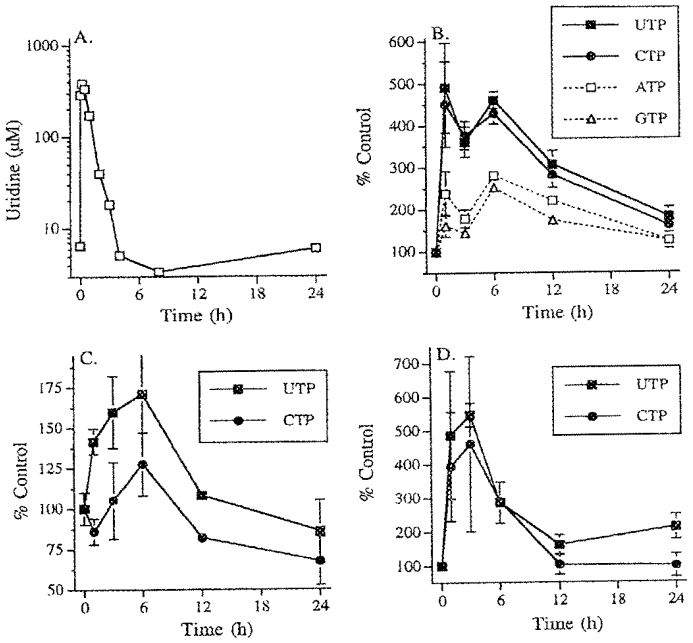

FIGURE 2. Pharmacokinetics of uridine after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. (A) Serum was harvested at the indicated times and serum uridine concentrations determined by HPLC. Data are presented as mean of 4–13 rats/group, and bars represent SEM. (B–D) Pharmacokinetics of pyrimidine and purine nucleotides in the spleen (B), liver (C) and lymph node (D) after a single, i.p., dose of uridine (500 mg/kg) in Lewis rats. Lewis rats were killed at the indicated times (4–6 rats/group), and the nucleotides extracted following protocols described under Materials and Methods. Data are presented as percentages of untreated controls, and bars represent SEM. The baseline concentrations of UTP, CTP, ATP, and GTP in the liver tissue were 92.0, 4.0, 357.7, 74.8 pg/mg; in the spleen tissue were 28.9, 21.0, 211.8, and 50.9 pg/mg, and in the lymph nodes tissues were 8.8, 7.3, 55.8, and 8.5 pM/20×10^6 cells, respectively.

treatment with 35 mg/kg/day resulted in the sacrifice of all Lewis recipients with beating allografts in <30 days after the transplant because of leflunomide-related toxicities. Uridine (500 mg/kg/day, i.p.) co-administration with leflunomide did not significantly alter allograft survival, and all the hearts were beating on day 50 in the Lewis recipients receiving 5 or 15 mg/kg/day leflunomide plus uridine (Table 2). In the 35 mg/kg/day leflunomide plus uridine group, the toxicity of leflunomide was significantly reduced and 4 of the 5 Lewis recipients were alive, with beating allografts on day 50 after the transplant (Table 2). One of the Lewis recipients in this combination-treatment group died on day 39, with a beating allograft.

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TABLE 2. Effect of uridine on the ability of leflunomide to prevent the rejection of allograft (brown Norway) hearts by Lewis rats[a]

Treatment	Allograft survival (d)	Mean (d)	Histological scores
None	6, 6, 7, 7, 7, 7, 7, 8	6.9±0.2	3A–3B (X8)
Lef (5)	>50 (X8)	>50	2, 2, 2, 2, 3A, 3A, 3A, 3B
Lef (5)+Uridine	>50 (X8)	>50	2, 2, 2, 3A, 3A, 3B, 3B, 3B
Lef (15)	>50 (X6)	>50	1A, 1A, 1A, 1A, 1B, 2
Lef (15)+Uridine	>50 (X6)	>50	1A, 1A, 1B, 2, 2, 2
Lef (35)	>13, >26, >28, >29, >29	>25.0±3.0	0, 0, 0, 1A, 1A
Lef (35)+Uridine	>37, >50 (X4)	>47.4±2.6	0, 1A, 1A, 1A, 1A

[a] Uridine was administered i.p. at 500 mg/kg (once a day), whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for 50 days. Survival of leflunomide-treated Lewis rats or grafts were calculated from the day of transplantation, and presented as mean±SE. Histological scores were determined when the rats were killed or at the end of the experiment (day 50 after the transplant).

[b] > indicates that Lewis rats died of leflunomide-induced toxicity with beating allografts, or were killed at the end of the experiment (day 50 after the transplant).

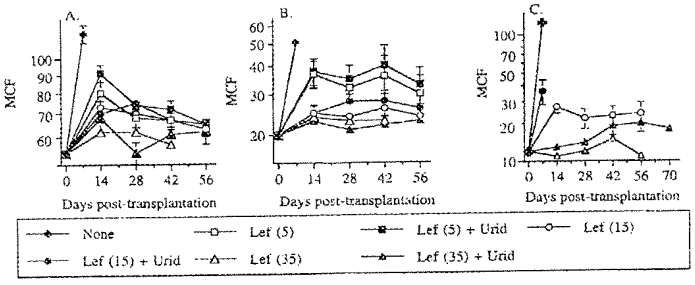

FIGURE 3. Levels of alloreactive IgM (A) and IgG (B) and xenoreactive IgM (C) in Lewis rats treated with leflunomide (Lef 5, 15, or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day) at the indicated days after the transplant. The relative amounts of alloreactive IgM and IgG and xenoreactive IgM were quantified by flow cytometric analysis, and data are presented as mean channel fluorescence (3 decade log scale)±SE. There were 3–6 rats per group.

We have previously reported that allograft rejection in this model is accompanied by an increase in the titers of alloreactive IgM and IgG (Fig. 3A, B). We here confirm that leflunomide, in a dose-dependent manner, inhibited the increase in both IgM and IgG titers. Co-administration of uridine resulted in a modest increase in the titers of alloreactive IgM and IgG in Lewis rats receiving a 5 mg/kg/day dose of leflunomide but had minimal effects at the higher doses of leflunomide.

On day 50 after the transplant, or at the time the rats were killed, the hearts were harvested and subjected to histological examination. Despite daily treatment with leflunomide (5 mg/kg/day) for 50 days, the allografts demonstrated a mild to moderately intense inflammatory infiltrate and some myocyte necrosis (data not shown). Cellular rejection was significantly reduced in allografts when the recipients were treated with a higher dose of leflunomide (15 mg/kg/day; Fig. 4A, B) and there were no signs of rejection in the allografts harvested from Lewis rats receiving the highest dose of leflunomide (35 mg/kg/day) at the time they were sacrificed (days 13–29 after the transplant; data not shown). In the group receiving uridine (500 mg/kg/day) and leflunomide (5, 15, or 35 mg/kg/day), only slightly exacerbated cellular rejection was observed in the allografts examined on day 50 after the transplant (Table 2, Fig. 4E, F). Some foci of infiltrating T cells and ED1 macrophages were observed, and marginally increased deposition of IgM in the leflunomide plus uridine groups compared to that in the leflunomide (15 mg/kg/day) monotherapy groups (Fig. 4E, F). There was minimal deposition of IgG in the allografts from all leflunomide monotherapy and leflunomide plus uridine groups (data not shown).

Leflunomide induced a dose-dependent reduction in the lymphoid compartments in the spleens of Lewis rats transplanted with Brown Norway hearts. The T lymphocyte zones of the periarteriolar lymphocyte sheath and the B cell zones, located in the follicles, marginal zones, and red pulp (especially around the pulp arterioles) were significantly reduced by leflunomide (15 mg/kg/day; Fig. 4C, D). Uridine completely reversed the effects of leflunomide on the lymphoid compartments in the spleen (Fig. 4G, H), suggesting that, although uridine is able to reverse the antiproliferative effects of leflunomide in the spleen, it has only modest effects on the ability of leflunomide to control allograft rejection.

Effect of uridine on the ability of leflunomide to control acute allograft rejection in C3H mice. Balb/c hearts transplanted into C3H mice were rejected in 8–10 days without immunosuppressive therapy (Table 3). Leflunomide at a dose of 30 mg/kg/day delayed rejection for 21–31 days (31.0±1.8). At this dose, there was no detectable toxicity in the mouse as a result of leflunomide administration. The lack of toxicity probably reflects the reduced sensitivity of mouse DHO-DHase to leflunomide compared with the rat enzyme (*2*, *3*, *21*, *22*). The mean percent of packed cell volume (PCV) in leflunomide-treated C3H mice, measured on the day of rejection, was 52.3±5.7, while in untreated controls was 62.0±2.8. Co-administration of uridine (500 mg/kg/dose; i.p., twice a day) with leflunomide resulted in a slightly more rapid rate of allograft rejection in 19–31 days (23.8±2.7; N=5). This was the maximum dose of uridine that could be administered in this experimental protocol as 4 of 9 mice died of uridine-related toxicity. The mean percent PCV in the surviving recipients, measured on the day of rejection, was 47.2±4.4%.

Effect of uridine on the ability of leflunomide to control acute xenograft rejection in Lewis rats. The rejection of hamster grafts by Lewis rats is mediated by anti-hamster IgM produced in a T-independent and T-dependent manner (*23–25*). We have previously reported that leflunomide at 15 mg/kg/day, but not at the 5 mg/kg/day dose, can prevent acute xenograft rejection in the hamster-to-Lewis transplantation model (*17*). Leflunomide at 15 mg/kg/day resulted in xenograft survival ranging from 48 to ≥75 days (mean graft survival was >63.2±9.7 days). When leflunomide was in-

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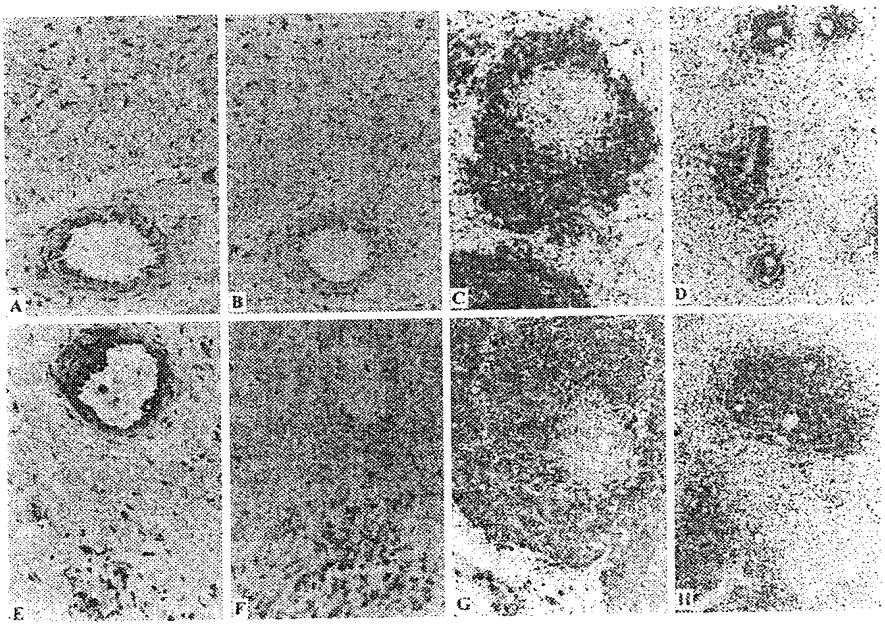

FIGURE 4. The ability of leflunomide (15 mg/kg/day) to inhibit allograft rejection (A and B) was minimally affected by uridine (500 mg/kg/day) co-administration (E and F). The proliferation of T and B cells in the recipient spleen in response to allograft stimulation was inhibited by leflunomide (15 mg/kg/day) (C and D), and this inhibition was reversed by uridine (500 mg/kg/day) co-administration (G and H). A and E: IgM deposition; B and F: TCR$\alpha\beta^+$ T lymphocytes; C and G: B cells (IgM^+) regions in the recipient spleen; D and H: TCR$\alpha\beta^+$ T cell regions in the spleen.

TABLE 3. Effect of uridine on the ability of leflunomide to control allograft (Balb/c) rejection in C3H mice[a]

Treatment	Allograft survival (d)	Means±SE
None	8, 9, 9, 10, 10	9.2±0.8
Uridine	8, 9, 9, 10, 11	9.4±1.1
Lef (30)	21, 27, 29, 30, 31	31.0±1.8
Lef (30)+Uridine	19, 19, 20, 30, 31	23.8±2.7

[a] Uridine was administered i.p. at 1000 mg/kg/day (500 mg/kg/dose given twice a day), whereas leflunomide was administered orally at 30 mg/kg daily from the day of the transplant until rejection. Data are presented as mean±SE. On days 5, 10, 12, and 13 after the transplant, 4 other recipients in this group, with functioning xenografts, died of uridine toxicity. This observation indicated that 1000 mg/kg/day is the maximum tolerable dose of uridine. The rest of the C3H recipients were killed on the day of graft rejection.

creased to 35 mg/kg/day, all the Lewis recipients died of, or were killed because of, leflunomide toxicity before the end of the experiment (75 days). The mean survival of the Lewis recipient was 36.0±14.0 days; however, at the time of death or sacrifice, all xenografted hearts were beating (Table 4).

We next tested the effect of uridine coadministration on the ability of leflunomide to prevent acute rejection in this transplantation model. Uridine 500 mg/kg/day coadministration completely antagonized the immunosuppressive activity of 15 mg/kg/day leflunomide, and the xenografts were rejected in 8.4±0.2 days. In contrast, co-administration of uridine 500 mg/kg/day and 35 mg/kg/day leflunomide resulted in long-term xenograft survival for up to 75 days in 3 of 5 recipients; the remaining 2 recipients were killed because of leflunomide toxicity with beating xenografts (Table 4).

As previously reported, pathological analysis of the xenografts on day 50 after the transplant revealed significant vascular injury, indicative of chronic rejection, when leflunomide was used at a dose of 15 mg/kg/day (*26, 27*). Xenografts removed after 10 days of leflunomide monotherapy (15 mg/kg/day) revealed minimal signs of rejection (Fig. 5A, B). A pathological examination of the grafts from rats treated with leflunomide plus uridine (average survival of 8.4 days) revealed severe acute rejection characterized by intense IgM deposition, arterial necrosis, thrombosis, and myocyte coagulating necrosis with a mild to moderate infiltrate comprising neutrophils and macrophages (Fig. 5E, F). At the time of sacrifice of Lewis rats treated with 35 mg/kg/day leflunomide (mean of 36.0±14.0 days after the transplant), the xenografts seemed histologically normal, with no signs of inflammatory cell infiltration or IgM deposition (Fig. 5C, D). Hearts from Lewis recipients treated with leflunomide (35 mg/kg/day) and uridine revealed mild mononuclear intracellular infiltration and IgM deposition, with minimal myocyte necrosis in 4 of 5 grafts (Fig. 5G, H). One graft had severe mononuclear cell infiltration with arterial intimal thickening, a feature characteristic of chronic rejection (data not shown).

We have previously reported that graft rejection in this concordant xenotransplantation model is accompanied by an increase in the titers of xenoreactive IgM but minimal increases in IgG (Fig. 3B) (*17*). We here confirm that leflunomide significantly inhibited the increase in xenoreactive IgM titers at a dose of 15 mg/kg/day and completely inhibited the increase in the xenoreactive IgM titers at the 35 mg/kg/day dose. Immunohistochemical analysis confirmed that leflunomide was able to inhibit xenoantibody production in a dose-dependent manner, with significant IgM deposition in the xenografts on day 75 after the transplant in groups treated with 15 mg/kg/day leflunomide (data not shown) (*26*), and minimal IgM deposition in the 35 mg/kg/day group (Fig. 5B and 5D).

In the groups in which uridine was co-administered with leflunomide, only marginally higher titers of circulating xenoreactive IgM were observed at the 15 mg/kg/day dose, and uridine had no detectable effect on the ability of the higher leflunomide dose to inhibit xenoantibody production. Immunohistochemical analysis indicated minimal IgM deposition in the xenografts from the leflunomide (15 mg/kg/day) monotherapy group on day 10 after the transplant (Fig. 5B), and dense IgM deposition in the xenograft at the time of rejection

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TABLE 4. Effect of uridine on the ability of leflunomide to control xenograft (hamster hearts) rejection in Lewis rats[a]

Treatment	Xenograft survival (d)	Mean (d)	Histological scores
None	3, 4, 4, 4, 4, 4, 4, 4	3.9±0.3	4 (AR; X9)
Lef (5)	6, 6, 7, 7, 7, 7, 8, 14	7.7±2.5	4 (AR; X9)
Lef (5)+Uridine	ND		ND
Lef (15)	48, 57, 57, 59, 60, 63, 75, >75[b] >75	>63.2±3.2	4 (CR; X9)
Lef (15)+Uridine	8, 8, 8, 9, 9	8.3±0.25	3B, 4, 4, 4 (AR)
Lef (35)	>24, >30, >31, >35, >60	>35.8±6.3	0, 0, 0, 0, 1A (AR)
Lef (35)+Uridine	>34, >34, >75, >75, >75	>58.3±10	1B, 1B, 1B, 2, 3B

[a] Uridine was administered i.p. at 500 mg/kg (once a day) whereas leflunomide was administered orally at 5, 15, or 35 mg/kg daily for up to 75 days. Survival of Lef-treated Lewis rats or grafts were calculated from the day of the transplant and presented as mean±SE. Xenografts were harvested on the day the rats were killed or at the end of experiment (day 75 after the transplant). Histological scores were determined as described under Materials and Methods.

[b] > indicates that the Lewis recipients died or were killed with beating grafts on day 75 after the transplant. AR, acute rejection; CR, chronic vascular rejection.

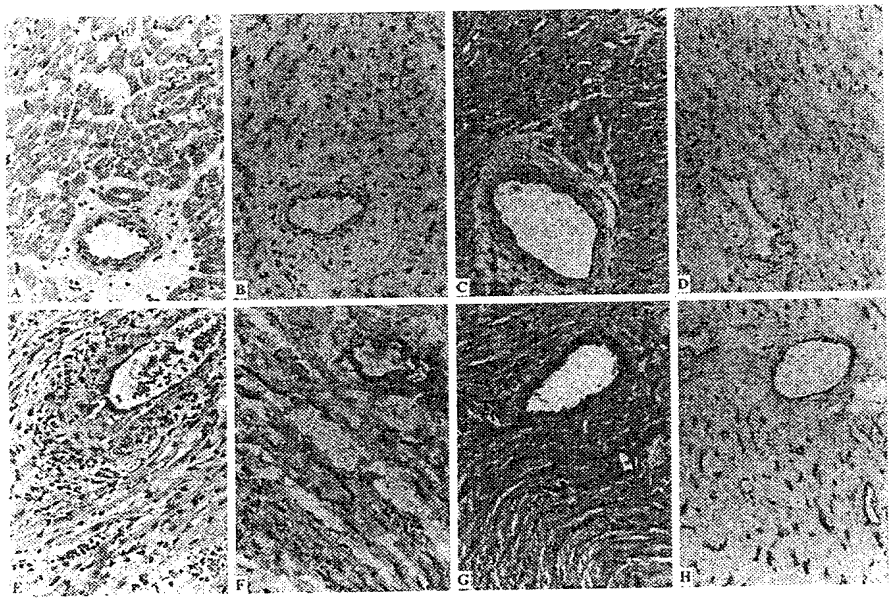

FIGURE 5. The ability of leflunomide (15 mg/kg/day (A, B, E, F) and 35 mg/kg/day) (C, D, G, H) to inhibit xenograft rejection was significantly reversed by uridine co-administration (E–H). Xenografts were harvested on day 10 after transplant (A and B), rejection (D and F), day of sacrifice (C and D), or day 75 after transplant (G and H). A, C, E, and G: HE staining; B, D, F and H: IgM immunostaining.

(day 8–10 after the transplant) in the group treated with 15 mg/kg/day of leflunomide plus uridine (Fig. 5F). There was also marginally more IgM deposition in the xenografts on day 75 in the groups treated with 35 mg/kg/day of leflunomide plus uridine (Fig. 5H), compared with the leflunomide monotherapy group (Fig. 5D). These results indicate that uridine can reduce the ability of leflunomide to control xenoreactive IgM production in the hamster-to-Lewis concordant xenotransplantation model.

Effect of uridine on the toxic side-effects of leflunomide in Lewis rats. Lewis rats with either an allograft or a xenograft, and treated with leflunomide at a dose of 35 mg/kg/day, survived for a mean of 30.5 days (Fig. 6A), with only one of 10 rats surviving <50 days. When the same dose of leflunomide was administered with uridine (500 mg/kg/day), the mean survival of Lewis rats was significantly enhanced, and 7 of 10 Lewis rats were alive after 50 days (Fig. 6A). These observations suggest that this dose of uridine could antagonize the toxic side effects of high-dose leflunomide. Lewis recipients treated with 15 mg/kg/day of leflunomide alone, or in combination with uridine, exhibited minimal signs of toxicity.

Typical signs of leflunomide toxicity in Lewis rats are anemia and diarrhea. We measured the percent of PCV every 14 days after the transplant in the Lewis rats receiving either allografts or xenografts. Treatment with 35 mg/kg/day of leflunomide resulted in a rapid decline in the percentage of PCV (Fig. 6B). In contrast, in the groups receiving uridine and 35 mg/kg/day of leflunomide, the decline in percent of PCV was delayed (Fig. 6B). In the Lewis recipients treated with 15 mg/kg/day leflunomide alone, or combination with uridine, there was no significant drop in the percentage of PCV for the duration of the experiment.

At the end of the experiment (either natural death or being killed), Lewis rats were subjected to a complete autopsy. Histological signs of leflunomide toxicity (35 mg/kg/day) were observed primarily in the small bowel and liver (Fig. 7A, B). Epithelial abnormalities were observed in the intestinal mucosa of the small bowel of rats treated with high-dose leflunomide (35 mg/kg/day). In particular, the villi were short and wide and mature intestinal epithelial cells, including goblet cells, brush border absorption epithelium, and Paneth's cells were partially or completely substituted by immature low columnar cells, with or without dysplasia (Fig. 7A). These epithelial abnormalities could be caused by atrophy, dedifferentiation, or inhibition of regeneration of the intestinal mucosa by high-dose leflunomide. Uridine significantly pre

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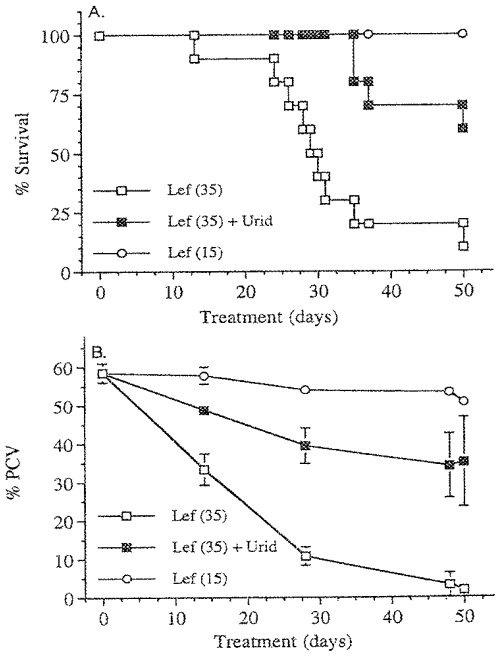

FIGURE 6. (A) Survival of Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). (B) The hematocrits in Lewis recipients after treatment with leflunomide (Lef; 15 or 35 mg/kg/day) alone or in combination with uridine (Urid; 500 mg/kg/day). Data are presented as mean PCV of 10 rats±SE.

vented changes in the small bowel in the three surviving Lewis rats treated with leflunomide and uridine for ≥75 days (Fig. 7C).

Toxicity in the liver was characterized by fatty degeneration, atrophy, and necrosis of the hepatocytes in the central lobular regions (Fig. 7B). These changes were completely abrogated with the co-administration of uridine, and the findings of the histological analysis of the liver seemed normal (Fig. 7D).

DISCUSSION

When relating the *in vitro* activity of a drug to its *in vivo* activity, it is usually necessary to assess the drug levels in the blood and tissue, at doses known to modify the function of the target organ system. With leflunomide, two *in vitro* activities are known to exist at two distinct concentrations: inhibition of *de novo* pyrimidine synthesis and selective inhibition of tyrosine kinases. Either of these activities might account for the immunomodulatory activity of leflunomide. To individually assess these activities *in vivo* requires that at least one of the activities be controlled. Uridine can be used as an antidote for inhibition of *de novo* pyrimidine synthesis; thus, we first conducted a pharmacokinetic analysis of serum A77 1726 after the administration of leflunomide, and of serum uridine and tissue nucleotide triphosphate levels after the administration of uridine.

After a single oral administration of leflunomide (5, 15, and 35 mg/kg), we observed a linear dose-dependent relationship between the dose administered and the concentration of A77 1726 in the serum. Consistent with previous studies, the increase in A77 1726 levels in the serum was relatively slow (Ka=1.97 to 0.484 mL · min/kg) and peak levels were reached in 6–8 hr, irrespective of the dose (*28*, *29*). After the administration of a single, i.p. dose 500 mg/kg of uridine in Lewis rats, increased levels of serum uridine were detected almost immediately and the peak concentration of uridine in the serum, 384.1±53.5 μM/L, was observed within 15 min (*28*, *29*). However, uridine plasma levels returned to normal by 4 hr, indicating that the pharmacokinetics of uridine are significantly different from that of A77 1726. The levels of UMP and CTP in the spleen, lymph nodes, and liver of Lewis rats were elevated within 1 hr after the administration of 500 mg/kg uridine. In contrast to rapid elimination of serum uridine, the levels of UTP and CTP in the spleen, liver, and lymph nodes remained elevated for 6–12 hr. Our data further suggest a hierarchy in the duration of elevated pyrimidine nucleotides in the spleen>liver>lymph nodes, perhaps reflecting the ability of different tissues or cells to salvage uridine and the availability of serum uridine (*30*, *31*). A

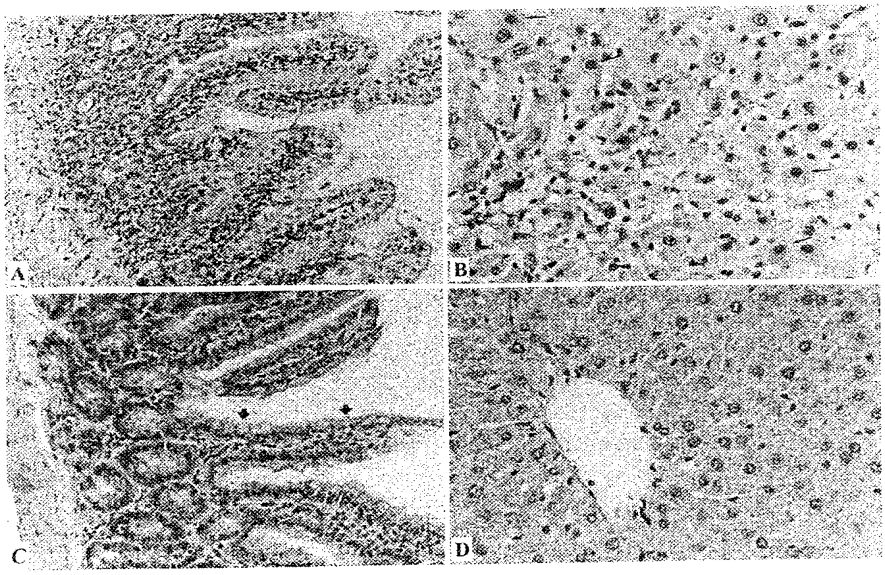

FIGURE 7. Histological examination of the effects of leflunomide (35 mg/kg/day) monotherapy (A, B), or in combination with uridine (C, D) in the small bowel (A, C), and liver (B, D). Liver and small bowel were stained with hematoxylin and eosin.

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critical role of the spleen in the development of cellular and antibody responses is suggested by the central position of the spleen in blood circulation and the large numbers of lymphocytes migrating. Thus, it is noteworthy that the pharmacokinetic findings of UTP levels after uridine administration in the spleen is most similar to that of serum A77 1726 after administration of leflunomide.

We next examined the effects of uridine on the immunosuppressive activity and toxicity of leflunomide in Lewis rats receiving with brown Norway or Golden Syrian hearts. On the basis of three criteria: survival, histological examination of the allograft, and titers of alloreactive antibodies, we conclude that the ability of leflunomide to control allograft rejection seems to be only minimally affected by uridine co-administration. It is interesting that the lymphoid areas in the spleen that were significantly reduced in leflunomide-treated rats receiving allograft hearts were significantly reversed by uridine co-administration. We, therefore, speculate that the immunosuppressive effects of leflunomide in this allotransplantation model are independent of the DHO-DHase-dependent antiproliferative effects of leflunomide. In a second allograft model (Balb/c into C3H), uridine also had modest effects on the immunosuppressive activity of leflunomide. These data are consistent with the conclusion that the mechanism by which leflunomide controls alloreactivity is largely independent of inhibition of pyrimidine synthesis *in vivo*.

The modest effect of uridine in this allograft model contrasts with our *in vitro* data that indicate that uridine can completely antagonize the antiproliferative activity of the active metabolite of leflunomide, A77 1726, when it is used at concentrations that are $\leq$25 μM (*9*). However, we had noted that uridine only partially reversed the antiproliferative effects of A77 1726 when the concentrations were $\geq$50 μM, and had no effect of the ability of A77 1726 to inhibit T cell cytotoxic activity (*9*). Because A77 1726, at IC_{50} of $\geq$50 μM, inhibits tyrosine phosphorylation in lymphocytes, we had hypothesized that the immunosuppressive activity at $\geq$50 μM A77 1726, and in the presence of uridine, results from inhibition of tyrosine phosphorylation (*6, 9, 32*).

Single-dose pharmacokinetic studies of rats treated with leflunomide at 5, 15, and 35 mg/kg/day indicated that peak concentrations of A77 1726 in the sera were 77.5, 266.1 and 478.8 μM, respectively, whereas the 24 trough concentrations are 4.9, 6.3, and 125 μM, respectively. Additionally, single-dose pharmacokinetic studies of mice treated with leflunomide at 35 mg/kg/day indicates that the peak concentration of A77 1726 in the sera is 518.2 μM, whereas the 24 hr trough concentration is 227.5 μM (data not shown). Therefore, the inability of uridine to counter the effects of leflunomide in this allograft model is consistent with the *in vitro* observations that uridine cannot reverse the immunosuppressive activities of higher doses of A77 1726 *in vitro* (*9*). We, thus, conclude that the primary mode of immunosuppression by leflunomide in this allograft model may be related to the inhibition of tyrosine phosphorylation and that the inhibition of *de novo* pyrimidine synthesis is of secondary importance.

In contrast to the modest effect of uridine in the allograft model, the ability of uridine to antagonize the immunosuppressive activity of 15 mg/kg/day of leflunomide in the xenograft model is very convincing. In the leflunomide monotherapy groups (15 mg/kg/day), the xenografts survive for a mean of >63 days. There was minimal IgM deposition in the xenograft on day 10 but significant IgM at the time of rejection or on day 75 after the transplant. In the combination therapy group, the xenografts were rejected in 8–10 days, and rejection was associated with extensive deposition of IgM in the xenograft. At the higher dose of leflunomide (35 mg/kg/day), the effect of uridine was more modest and the xenograft hearts were still beating at the time the rats were killed (day 34 or day 75 after the transplant). A histological examination of the xenografts from the combination treatment group (leflunomide [35 mg/kg/day] plus uridine) revealed increased signs of inflammation, chronic rejection, and IgM deposition, compared with the 35 mg/kg/day leflunomide monotherapy group. These observations suggest that inhibition of *de novo* pyrimidine synthesis is an important part of immunosuppressive therapy in the xenotransplantation model. It further suggests that insufficient uridine is not the explanation for our observations in the allotransplantation model.

The contrasting effect of uridine on the immunosuppressive activity of leflunomide may result from different mechanisms of rejection in the allograft versus the xenograft model. Acute xenograft rejection is dependent on the rapid production of xenoreactive IgM; in contrast, acute allograft rejection is a T-cell dependent process (*17, 33*). Therefore, it is possible that the control of B cell function in xenograft rejection by leflunomide depends more on the inhibition of pyrimidine synthesis, whereas the control of T cells by leflunomide may be more dependent on the inhibition of tyrosine phosphorylation. *In vitro* observations suggest that B cells may be more susceptible to the effects of inhibition of *de novo* pyrimidine synthesis than T cells (*9, 10, 34–36*).

A second goal of these studies was to investigate whether uridine could be used to control leflunomide-induced toxicity. The most consistent symptoms in Lewis rats treated with 35 mg/kg/day of leflunomide were severe anemia and diarrhea resulting in weight loss, dehydration and, ultimately, death. Uridine was able to significantly reduce the anemia and prolong the survival of the Lewis rats. Autopsies revealed liver necrosis and pathological changes in the small bowel in rats treated with 35 mg/kg/day leflunomide. Most of these changes were significantly reversed by uridine co-administration, suggesting that the toxicity of the liver and small bowel, and the inhibition of hematopoiesis, is largely caused by inhibition of pyrimidine synthesis.

In summary, we report that uridine had minor effects on the immunosuppressive activity of leflunomide in the allograft model, and a more significant effect in the xenograft model. Thus, it seems that the mechanism of immunosuppression by leflunomide *in vivo* is complex and may be affected by at least the following four factors: the type and vigor of the immune response; the availability of uridine for salvage by proliferating lymphocytes; the species-specific efficacy by which leflunomide inhibits the activity of dihydroorotate dehydrogenase, and the levels of A77 1726 *in vivo*.

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Uridine Reverses the Toxicity of 3'-Azido-3'-Deoxythymidine in Normal Human Granulocyte-Macrophage Progenitor Cells In Vitro without Impairment of Antiretroviral Activity

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We evaluated the effects of natural purine and pyrimidine nucleosides on protection from or reversal of 3'-azido-3'-deoxythymidine (AZT) cytotoxicity in human bone marrow progenitor cells by using clonogenic assays. The selectivity of the "protection" or "rescue" agents was examined in evaluating the antiretroviral activity of AZT in combination with these modulating agents and of AZT alone. Following exposure of human granulocyte-macrophage progenitor cells for 2 h to 5 μM AZT (70% inhibitory concentration), increasing concentrations of potential rescue agents were added. Cells were cultured, and colony formation was assessed after 14 days. At concentrations of up to 50 μM no natural 2'-deoxynucleosides, including thymidine, were able to reverse the toxic effects of AZT. Dose-dependent reversal was observed with uridine and cytidine, and essentially complete reversal was achieved with 50 μM uridine. In the protection studies, 100 μM thymidine almost completely antagonized the inhibition of granulocyte-macrophage colony formation produced by 1 μM AZT (50% inhibitory concentration), and 50 μM uridine effected 60% protection against a toxic concentration of AZT (5 μM) (70% inhibitory concentration). The antiretroviral activity of AZT in human peripheral blood mononuclear cells, assessed by reverse transcriptase assays, was substantially decreased in the presence of thymidine, whereas no impairment of suppression of viral replication was observed in the presence of uridine in combination with AZT at a molar ratio (uridine/AZT) as high as 10,000. This demonstration of the capacity of uridine to selectively rescue human bone marrow progenitor cells from the cytotoxicity of AZT suggests that use of uridine rescue regimen with AZT may have potential therapeutic benefit in the treatment of acquired immunodeficiency syndrome.

3'-Azido-3'-deoxythymidine (AZT), a pyrimidine nucleoside synthesized two decades ago by Horwitz et al. (7), has recently been shown to transiently improve certain immunological functions in some patients with acquired immunodeficiency syndrome (AIDS) (3), resulting in a decrease in the incidence of opportunistic infections and prolonging survival. The antiretroviral effects of AZT are probably based upon its conversion through cellular kinases to AZT triphosphate, which binds to reverse transcriptase and thereby inhibits viral DNA synthesis by chain termination (4). Although AZT selectively inhibits the replication of human immunodeficiency virus type 1 (HIV) (10), its applications in preliminary clinical trials (11, 15) were limited by expressions of bone marrow toxicity. Consistent with these expressions we recently reported (12) that continuous exposure to AZT for 14 days effected a dose-dependent inhibition of human granulocyte-macrophage CFU (CFU-GM) and erythroid burst-forming unit colonies, the 50% inhibitory concentrations being 0.9 ± 0.1 and 2.4 ± 0.4 μM for the respective colonies. Several pharmacologic approaches are potentially available to improve the chemotherapeutic selectivity of AZT. Such an improvement can be theoretically obtained with synergistic combination chemotherapy, as recently demonstrated with recombinant human granulocyte-macrophage colony-stimulating factor (5) and alpha A interferon (6), potentially allowing a reduction in AZT concentrations. Selective "protection" or "rescue" combinations may also achieve this goal. In this approach, the modulating agents are used at a time and a dosage that counteract (protection) or reverse (rescue) the toxic effects in the host cell without interfering with the chemotherapeutic activity. This concept has been previously used in cancer chemotherapy with methotrexate and its "antidote," leucovorin (2), and more recently for treating protozoan infections with trimetrexate and leucovorin in AIDS patients (1).

The present report relates the results of an evaluation of the capacities of various natural nucleosides to protect or to reverse AZT toxicity in human host cells. The selectivity of the metabolic modulations was assessed by evaluating their antiretroviral activity in comparison with that of AZT alone in HIV-infected cells.

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MATERIALS AND METHODS

Chemicals. Purine and pyrimidine ribonucleosides and deoxyribonucleosides were purchased from Sigma Chemical Co., St. Louis, Mo. AZT was synthetized in our laboratory by the procedure of Lin and Prusoff (8) and had a purity of >99%, as assessed by high-pressure liquid chromatography. The structure of the compound was confirmed by proton nuclear magnetic resonance, ^{13}C nuclear magnetic resonance, and infrared spectroscopy. Other chemicals were of the highest quality commercially possible.

Virus strains. HIV strain LAV was obtained from the

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Centers for Disease Control, Atlanta, Ga., and propagated as recently described (13).

Preparation of cells. Human bone marrow cells were collected by aspiration from the posterior iliac crest of normal healthy volunteers and treated with heparin, and the mononuclear population was separated by Ficoll-Hypaque gradient centrifugation. Cells were washed twice in Hanks balanced salt solution and counted with a hemacytometer, and their viability was >98%, as assessed by trypan blue exclusion. Peripheral blood mononuclear (PBM) cells were obtained from the whole blood of healthy HIV- and hepatitis B virus- seronegative volunteers and collected by single-step Ficoll-Hypaque discontinuous gradient centrifugation.

Assay of CFU-GM for drug cytotoxicity and rescue or protection studies. The culture assay of CFU-GM was performed by a bilayer soft-agar method as recently described (12). McCoy 5A nutrient medium supplemented with 15% dialyzed fetal bovine serum (heat inactivated at 56°C for 30 min) (GIBCO Laboratories, Grand Island, N.Y.) was used in all experiments. This medium completely lacked thymidine and uridine.

In the rescue studies, mononuclear cells (10^5/ml) were exposed for 2 h at 37°C in 5 ml of McCoy 5A nutrient medium to 5 μM AZT, corresponding to a 70% inhibitory concentration. At the end of the 2-h incubation period, cells were washed twice with fresh cold incubation medium to wash out the AZT. Cells were subsequently cloned in 0.3% agar in the presence of increasing concentrations of the modulating compound or in medium alone (control). After 14 days of incubation at 37°C in a humidified atmosphere of 5% CO_2 in air, colonies (≥50 cells) were counted by using an inverted microscope.

In the protection studies, AZT (1 or 5 μM) and either medium (control) or various concentrations of thymidine or uridine were added simultaneously. Cells were exposed continuously under these conditions for 14 days, and colonies (≥50 cells) were then scored. The toxicity of each purine and pyrimidine analog investigated in these studies (see Table 1) was assessed by continuous exposure for 14 days by the same technique.

Anti-HIV assay based on reverse transcriptase activity. After phytohemagglutinin stimulation for 3 days, PBM cells (5×10^5/ml) were infected with HIV strain LAV at a concentration of about 100 50% tissue culture infective doses per ml and cultured in the presence of various concentrations of AZT alone or in combination with either uridine or thymidine. The virus was allowed to adsorb for 45 min, and then drugs (AZT and uridine or thymidine) were added. A virus control (no drug) and a cell control (no virus or drug) were also included. On day 5 after infection, clarified supernatant fluids were centrifuged in a Beckman 70.1 Ti rotor at 40,000 rpm for 30 min. The concentrated, disrupted virus was subjected to reverse transcriptase assays as recently described by Spira et al. (13). Antiretroviral efficacy was determined by calculating the percent reduction in reverse transcriptase activity observed in drug-treated, virus-infected cultures as compared with enzyme activity in virus-infected control cultures.

RESULTS

Effect of a short exposure (2 h) of normal human bone marrow progenitor cells to AZT on colony growth. Initial experiments were designed to establish the concentration dependence of human bone marrow progenitor cell toxicity produced by AZT after 2 h of drug exposure. Normal human bone marrow cells were incubated at 37°C for 2 h with various concentrations of AZT, and cells were washed twice prior to plating. Cell viability was determined by soft-agar cloning and measurement of colony formation after drug treatment as described in Materials and Methods. After 2 h of drug exposure, the toxic effects of AZT (Fig. 1) were quite similar to those recently reported for these cells after continuous exposure (14 days) to AZT (12), suggesting that the toxicity of AZT in human bone marrow progenitor cells in vitro is probably mediated through early effects.

Ability of purine or pyrimidine derivatives to reverse the toxicity of AZT in human bone marrow cells. The effects of

TABLE 1. Reversal of AZT cytotoxicity in human bone marrow progenitor cells by naturally occurring purine and pyrimidine nucleosides

Compound and concn (μM)	Survival (% of untreated control)[a] in the presence of compound:	
	Alone	With AZT (5 μM)[b]
Thymidine		
0	100	22.8 ± 7.8
5	ND[c]	24.6 ± 7.1
10	85.4 ± 3.1	24.6 ± 8.4
50	84.7 ± 14.1	17.7 ± 7.5
Cytidine		
0	100	22.8 ± 7.8
5	ND	25.3 ± 12.4
10	94.0 ± 7.3	23.5 ± 5.3
50	93.3 ± 6.2	50.0 ± 14.0[d]
Uridine		
0	100	22.8 ± 7.8
5	ND	24.5 ± 13.6
10	85.4 ± 3.1	43.2 ± 14.4[e]
50	84.7 ± 14.1	100.7 ± 20.3[d]
100	ND	85.6 ± 10.4[d]
2′-Deoxyuridine		
0	100	33.0 ± 16.0
5	ND	37.0 ± 10.4
10	100 ± 8.0	34.0 ± 6.0
50	91.3 ± 2.3	35.0 ± 10.5
2′-Deoxyadenosine		
0	100	33.0 ± 16.0
5	ND	36.0 ± 7.0
10	66.7 ± 5.5	37.3 ± 15.5
50	63.3 ± 6.4	42.3 ± 8.1
2′-Deoxyguanosine		
0	100	33.0 ± 16.0
5	ND	33.6 ± 16.2
10	83.4 ± 3.8	30.0 ± 6.2
50	ND	40.0 ± 6.0
2′-Deoxycytidine		
0	100	33.0 ± 16.0
5	ND	28.0 ± 7.0
10	82.7 ± 12.6	34.0 ± 10.4
50	94.6 ± 4.6	35.0 ± 4.6

[a] Each value represents the mean ± standard deviation in at least three experiments with at least three different marrow donors.
[b] Cells were incubated with AZT (5 μM) for 2 h, washed twice, and cultured for clonal growth for 2 weeks in the presence of purine or pyrimidine analogs.
[c] ND, Not determined.
[d] $P < 0.001$ as compared with the control.
[e] $P < 0.01$ as compared with the control.

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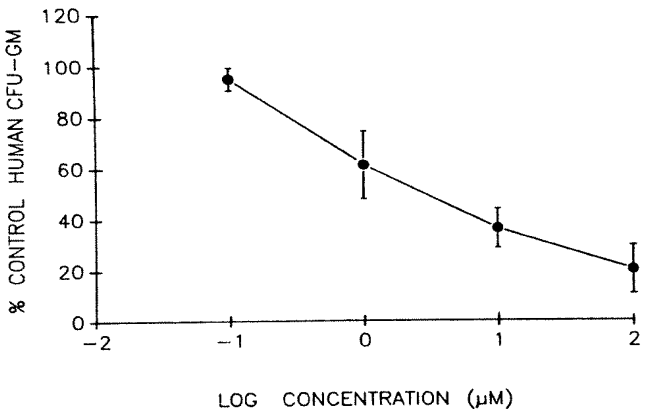

FIG. 1. Effects of a short exposure (2 h) of normal human bone marrow progenitor cells to increasing concentrations of AZT. Each point represents the mean ± standard deviation of at least six experiments with different marrow donors.

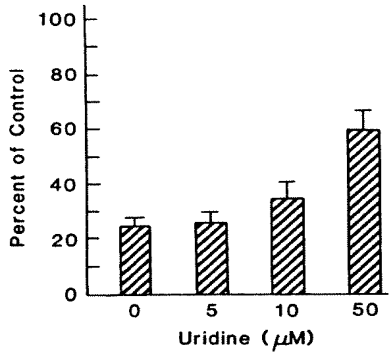

FIG. 2. Effect of AZT (5 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of uridine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and uridine) was 60 ± 5 CFU-GM per 10^5 cells. P was <0.01 for 10 μM uridine and <0.001 for 50 μM uridine as compared with the control.

adding purine or pyrimidine nucleoside analogs to human hematopoietic progenitor cells following 2 h of exposure to 5 μM AZT (70% inhibitory dose) are shown in Table 1. No natural purine or pyrimidine 2'-deoxynucleosides (2'-deoxyuridine, 2'-deoxyadenosine, 2'-deoxyguanosine, 2'-deoxycytidine, and thymidine) up to a concentration of 50 μM reversed the toxic effects of AZT, nor was reversal achieved with 2'-deoxynucleoside concentrations of up to 200 μM (data not shown). In contrast, nontoxic concentrations of uridine or cytidine effected a significant and dose-dependent reversal of AZT toxicity. Essentially complete reversal was achieved with 50 μM uridine, and no significant difference was detected in the presence of higher concentrations of uridine (100 μM). At concentrations of 50 μM the rescue effect of cytidine was less than that of uridine. The reversal of AZT toxicity by cytidine probably depends on the conversion of cytidine to uridine by cytidine deaminase, a requirement that could explain the difference in the rescue potencies of the two pyrimidine derivatives.

Protection from AZT toxicity by uridine in human bone marrow cells. Simultaneous exposure to 5 μM AZT (70% inhibitory dose) and various concentrations of uridine was also investigated in our studies to assess whether uridine could protect human bone marrow progenitor cells from AZT toxicity when both drugs were present throughout the experiment. Figure 2 illustrates the effects of 5 μM AZT on hematopoietic colony growth of normal human bone marrow progenitor cells in the presence of 5 to 50 μM uridine. Dose-dependent protection was observed, with 50 μM uridine effecting approximately 60% protection in the presence of a toxic concentration of AZT (5 μM).

Protection from AZT toxicity by thymidine in human bone marrow cells. Since thymidine counteracts the antiretroviral activity of AZT in ATH8 cells (10) and both AZT and thymidine appear to share the same activating enzymes (i.e., thymidine and thymidylate kinases) (4), protection from AZT toxicity by thymidine was investigated in human bone marrow progenitor cells. Human granulocyte-macrophage precursor cells, grown in soft agar, were exposed continuously for 14 days to 1 μM AZT (50% inhibitory dose) and 10 to 100 μM thymidine. The latter agent antagonized the inhibition of colony formation by AZT in a dose-dependent manner, almost complete protection being effected by 100 μM thymidine (Fig. 3).

Effect of thymidine and uridine on the antiretroviral activity of AZT in HIV-infected human PBM cells. The effects of thymidine and uridine on the capacity of AZT to inhibit HIV replication in human PBM cells was evaluated (Table 2). Essentially full protection against HIV production was achieved with AZT alone at a concentration of approximately 0.01 μM, in agreement with recently published data (9). The presence of thymidine led to a substantial loss of the antiretroviral activity of AZT, as reported previously (10). A concentration of 10 μM thymidine reduced the inhibition of HIV by 0.01 μM AZT by approximately 50%, and the anti-HIV activity of 0.01 μM AZT was essentially abolished by 100 μM thymidine. In contrast, concentrations of uridine of up to 100 μM and in combination with AZT at a molar

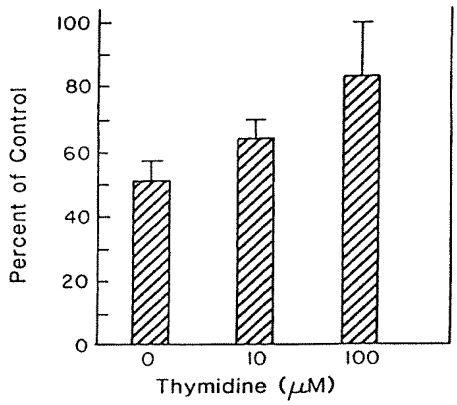

FIG. 3. Effect of AZT (1 μM) on hematopoietic colony growth by normal human bone marrow progenitor cells in the presence of various concentrations of thymidine. Columns represent the mean percentage of inhibition of CFU-GM colonies in three separate experiments; bars represent the standard deviation. The mean number of colonies in the control plates (without AZT and thymidine) was 80 ± 20 CFU-GM per 10^5 cells.

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TABLE 2. Effect of thymidine and uridine on AZT antiretroviral activity in HIV-infected human PBM cells

Treatment and concn (μM)	% Inhibition on day 5[a] (corrected)
AZT	
0.001	0
0.01	81.9
0.1	96.6
Thymidine	
10	0
100	0
Uridine	
10	7.3
100	5.1
AZT-thymidine	
0.01-0.1	81.4
0.01-1	85.0
0.01-10	52.6
0.01-100	4.1
AZT-uridine	
0.01-10	75.1
0.01-100	63.2
0.1-10	96.7
0.1-100	96.1

[a] The mean of triplicate counts (± standard deviation) for the virus control was 234,780 ± 26,600 dpm/ml (equivalent to an approximate incorporation of 3.6 pmol of dTMP into the acid-unsoluble product). Counts for the blank and negative control (no virus or drug) were 355 and 1,535 dpm, respectively. A positive control for the reverse transcriptase assay was also included (count, 313,000 dpm).

ratio (uridine/AZT) as high as 10,000 had no effect on the antiretroviral activity of AZT. Cell viability and growth, as assessed by trypan blue exclusion and with a Coulter Counter, were similar among the cultures, and no toxicity was observed in the presence of 100 μM uridine (Table 3).

TABLE 3. Effect of AZT and uridine alone or in combination on human PBM cell growth and cell viability[a]

Treatment and concn (μM)	Growth (% of untreated control)	Viability (% of untreated control)
AZT		
0.1	102.9	113.2
1	100	90.6
10	81.2	100
100	79.4	103.1
Uridine		
1	100	109.4
10	94.1	100
100	87.0	100
AZT-uridine		
1-1	97.1	78.8
1-10	105.9	72.5
1-100	78.1	78.2

[a] The mean numbers of human PBM cells (± standard deviation) in the cell growth and viability studies were (3.2 ± 0.6) × 10^6 cells per ml and 3.4 ± 0.3 cells per ml, respectively. Cells were stimulated with phytohemagglutinin for 2 days and subsequently exposed for 5 days to various concentrations of AZT or uridine alone or in combination.

These data suggest that, unlike thymidine, uridine does not interfere with the uptake and/or metabolic activation of AZT in HIV-infected human PBM cells or any other mechanism(s) by which AZT inhibits HIV replication.

DISCUSSION

In a previous report from this laboratory, we demonstrated that AZT directly suppressed human hematopoietic colony growth in a dose-dependent manner by direct interaction with CFU-GM and erythroid burst-forming unit progenitor cells (12). These findings were consistent with the observation that anemia and neutropenia were the major adverse effects of AZT administration to AIDS patients (11, 15). In an effort to obviate this untoward effect of AZT, we have sought to determine in the present study whether natural nucleosides can protect against or reverse the toxicity of AZT in human bone marrow progenitor cells without affecting the antiretroviral activity of AZT. As reported here, thymidine can counteract or protect against the toxicity of AZT in normal human bone marrow cells, but it also antagonizes the antiretroviral activity of AZT (Table 2). These data probably reflect a decrease in the formation of AZT triphosphate, since both AZT and thymidine utilize the same activating enzymes (i.e., thymidine and thymidylate kinases) to exert their pharmacologic action. It is particularly important that thymidine, even at concentrations of up to 100 μM, was not able to reverse the toxic effects of 5 μM AZT (70% inhibitory concentration) for human granulocyte-macrophage precursor cells. This result suggests that the administration of thymidine sequentially with AZT in vivo probably will not prevent its toxic effects, as previously speculated (11).

Our experiments also showed that the hematopoietic toxicity of AZT was consistently reversed by uridine and to a lesser extent by cytidine. The inhibition of CFU-GM colony formation at an AZT concentration of 5 μM was essentially reversed when 50 μM uridine was added to the cultures. The percentage of rescue of CFU-GM colony formation was proportional to the concentration of uridine, suggesting that the reversal was a competitive process. The same concentration of cytidine only partially reversed the toxic effects of AZT on colony formation. In these studies, cytidine probably acted through its conversion to uridine by cytidine deaminase, explaining the quantitative difference in the rescue between the two pyrimidine derivatives. Uridine was shown also to protect normal human bone marrow progenitor cells from AZT toxicity, and 60% protection was achieved when cells were exposed to both 5 μM AZT and 50 μM uridine for 14 days.

No difference in the inhibition of viral replication was observed in HIV-infected PBM cells when uridine was combined with AZT at different molar ratios as compared with AZT alone. Therefore, the combination of AZT and uridine appears to selectively reverse the hematopoietic effects of AZT without decreasing its antiretroviral activity. Uridine pharmacokinetic and toxicity studies have been recently reported in humans (14), and "rescuing" concentrations of uridine may be achieved in vivo, with a tolerable toxicity, making this combination potentially suitable for the treatment of AIDS. A potential mechanism(s) which may account for the reversal of or protection from AZT cytotoxicity by uridine in human bone marrow cells is unclear, and further investigations are currently in progress.

In summary, the high degree of selectivity of the uridine rescue between human bone marrow progenitor cells and

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HIV-infected cells suggests that the combined use of AZT and uridine may be of importance in the treatment of AIDS. Although the results of the present in vitro studies must be cautiously extended to the clinical situation, the possible use of uridine for rescue may have a potential therapeutic benefit in that the antiretroviral activity of AZT is not affected while the host toxicity of AZT is minimized. This novel strategy for modulating AZT therapy deserves further biochemical and/or pharmacologic investigations, which may lead to carefully controlled clinical evaluations.

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Uridine in the prevention and treatment of NRTI-related mitochondrial toxicity

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Long-term side effects of antiretroviral therapy are attributed to the mitochondrial (mt) toxicity of nucleoside analogue reverse transcriptase inhibitors (NRTIs) and their ability to deplete mtDNA. Studies in hepatocytes suggest that uridine is able to prevent and treat mtDNA depletion by pyrimidine NRTIs [zalcitabine (ddC) and stavudine (d4T)] and to fully abrogate hepatocyte death, elevated lactate production and intracellular steatosis. Uridine was also found to improve the liver and haematopoietic toxicities of zidovudine (AZT), which are unrelated to mtDNA depletion, and to prevent neuronal cell death induced by ddC. Most recently, uridine was found to prevent the onset of a lipoatrophic phenotype (reduced intracellular lipids, increased apoptosis, mtDNA depletion and mt depolarization) in adipocytes incubated long-term with d4T and AZT. Various steps of mt nucleoside utilization may be involved in the protective effect, but competition of uridine metabolites with NRTIs at polymerase γ or other enzymes is a plausible explanation. Pharmacokinetic studies suggest that uridine serum levels can be safely increased in humans to achieve concentrations which are protective *in vitro* (50–200 μM). Uridine was not found to interfere with the antiretroviral activity of NRTIs. Mitocnol, a sugar cane extract which effectively increases uridine in human serum, was beneficial in individual HIV patients with mt toxicity and is now being tested in placebo-controlled randomized trials. Until these data become available, the risk–benefit calculation of using uridine should be individualized. The current safety data justify the closely monitored use of uridine in individuals who suffer from mt toxicity but who cannot be switched to less toxic NRTIs.

Introduction

More than 8 years after the widespread introduction of highly active antiretroviral therapy (HAART), it has become clear that antiretroviral drugs have long-term effects on organs and body metabolism. Nucleoside reverse transcriptase inhibitors (NRTIs) within the antiretroviral cocktail are associated with hyperlactataemia and organ toxicities such as damage to the liver, peripheral nerves and skeletal muscle. The choice of NRTI also determines an individual's risk of developing lipoatrophy, a clinically irreversible loss of subcutaneous tissue. The main mechanism of these NRTI-related side effects has been identified as mitochondrial (mt) toxicity [1–7].

Pathogenesis of NRTI-related mt toxicity

NRTIs are activated by triphosphorylation and then they inhibit polymerase γ, the enzyme which replicates mtDNA [3,8]. Polymerase γ inhibition is a result of several distinct steps [3]. The first step involves competition of NRTI triphosphates with the natural nucleoside triphosphates. If this competition is successful, the NRTIs are incorporated into the nascent mtDNA strand. This second step causes chain termination. As a result of polymerase γ impairment, mtDNA depletion (a quantitative reduction of the mtDNA copy number) ensues. The relative potency of activated nucleoside triphosphates to inhibit polymerase γ is not the same among all NRTIs. *In vitro* data indicate a relatively strong inhibitory effect of the 'd-drugs', that is, zalcitabine (ddC), didanosine (ddI) and stavudine (d4T), whereas abacavir, emtricitabine, lamivudine and tenofovir do not impair mtDNA replication in clinically relevant concentrations [3,8,9].

Zidovudine (AZT) is a special case because this NRTI is a mt toxin despite the fact that AZT triphosphate only has a low potency to affect polymerase γ and mtDNA content in clinically relevant and cytotoxic concentrations, at least in proliferating cells [8–11]. On one hand, the mt toxicity of AZT may, in part, involve binding to adenylate kinase (an enzyme involved in ATP formation) and inhibition of the mt ADP/ATP translocator [12–14]. These mechanisms may explain why some toxicities have been observed relatively early after AZT exposure [9,13]. On the other hand, mtDNA depletion has indeed been

observed with AZT *in vivo* [6,15–17]. Two observations may explain why mtDNA depletion may also occur in the absence of direct polymerase γ inhibition. Firstly, it has been shown *in vivo* that some of the administered AZT can be non-enzymatically converted into d4T, and thus a stronger polymerase γ inhibitor [18]. Secondly, mtDNA depletion may result from a another mechanism, namely from AZT-mediated inhibition of thymidine kinase (TK) type 2 [19]. This TK is expressed in mitochondria and responsible for the intramitochondrial phosphorylation of pyrimidine nucleosides (deoxythymidine, deoxycytidine and deoxyuridine). In non-replicating cells, the cytosolic TK type 1 (TK1) is down-regulated, making the pyrimidine supply for mtDNA synthesis dependent on the activity of TK2. Such reduced supply of the normal deoxypyrimidine phosphates limits mtDNA replication, especially in skeletal muscle, as evidenced by a mt myopathy in subjects carrying TK2 mutations [20].

As mtDNA encodes for subunits of the mt respiratory chain, mtDNA depletion therefore results in respiratory chain dysfunction.

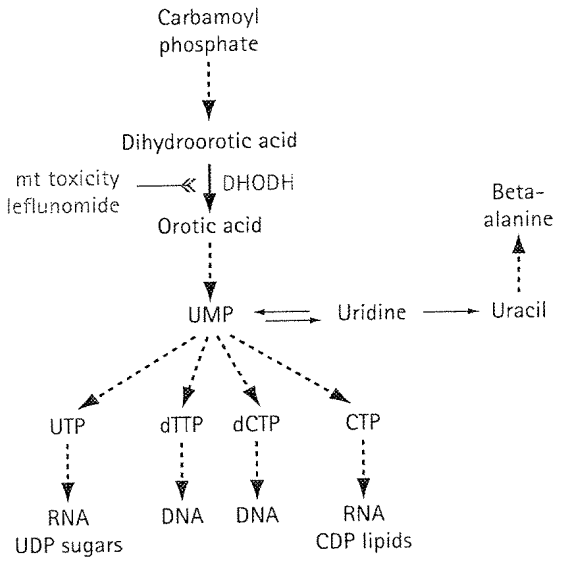
Any respiratory chain dysfunction may promote electron leakage in the mt matrix and thus the generation of reactive oxygen species (ROS). Such increased ROS formation may then in turn damage the lipid architecture of the mt membrane, attack respiratory chain proteins or damage polymerase γ and mtDNA itself, thereby closing several vicious circles that promote even more ROS formation [21,22]. There is also evidence for additional mechanisms of ROS formation [1]. Markers of oxidative damage and heteroplasmic mtDNA point mutations have indeed been shown to increase in patients treated with NRTIs [23,24].

Respiratory chain dysfunction also leads to the secondary impairment of several metabolic pathways. Firstly, ATP can no longer be synthesized efficiently through oxidative phosphorylation and glycolysis has to be relied upon. Secondly, the block of NADH utilization in the respiratory chain increases the intracellular NADH/NAD+ ratio. This alteration of the redox status promotes the conversion of pyruvate to lactate and inhibits key enzymes of beta oxidation, resulting in the intracellular accumulation of triglycerides [25].

The mt respiration also has a third important task: an efficient electron-flux through the respiratory chain is essential for the activity of dihydroorotate-dehydrogenase (DHODH; E.C. 1.3.99.11), an enzyme located in the inner mt membrane and necessary for the *de novo* synthesis of all (intramitochondrial and intracytoplasmic) pyrimidines [26]. This is because DHODH catalyses the oxidation of dihydroorotate to orotate from which uridine monophosphate (UMP) and intracellular pyrimidines are synthesized (Figure 1). A defect in the respiratory chain therefore results in pyrimidine depletion. The indirect inhibition of DHODH by NRTI-related mt toxicity is likely to be similar to those caused by direct DHODH inhibitors [27]. Research into leflunomide [27,28], a direct DHODH inhibitor and a licensed immunosuppressive drug has taught us about the *in vitro* and *in vivo* consequences of DHODH inhibition (Figure 2). The depletion of UMP and derived intracellular pyrimidines activates p53 and its immediate transcriptional target p21 [27,29]. p53 also regulates the activation of Rb protein and thus of cyclins via phosphorylation [30]. Through this mechanism, the pyrimidine depletion inhibits the transition to the S-phase of the cell cycle and leads to a mitotic arrest in the G1 phase. p53 can also activate the transcription of *Bax* [31] and promote apoptosis. These molecular mechanisms may explain why cells with mtDNA depletion stop dividing and then die.

The importance of the intracellular pyrimidine pools for the survival of cells without a functional respiratory chain is supported by the fact that cells without a single molecule of mtDNA (rho0-cells) are rescued from cell

Figure 1. Simplified scheme of pyrimidine metabolism



The biosynthetic pathway starts with the formation of carbamoyl phosphate. DHODH (an enzyme which is inhibited by respiratory chain dysfunction in mt toxicity of NRTIs and by leflunomide) then catalyses the synthesis of orotate. Orotate is then anabolized to UMP, which can be used to produce RNA, DNA, glycosylation products or membrane constituents. Uridine can be salvaged into UMP by uridine kinase or degraded into beta-alanine, which enters the tricarboxylic acid cycle. Dashed arrows signify pathways involving intermediate metabolites. mt, mitochondrial; CDP, cytidine diphosphate; DHODH, dihydroorotate dehydrogenase; dCTP, dideoxycytidine triphosphate; dTTP, dideoxythymidine triphosphate; UDP, uridine diphosphate; UMP, uridine monophosphate; UTP, uridine triphosphate.

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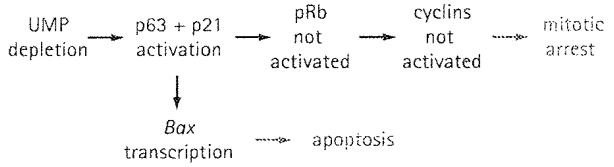
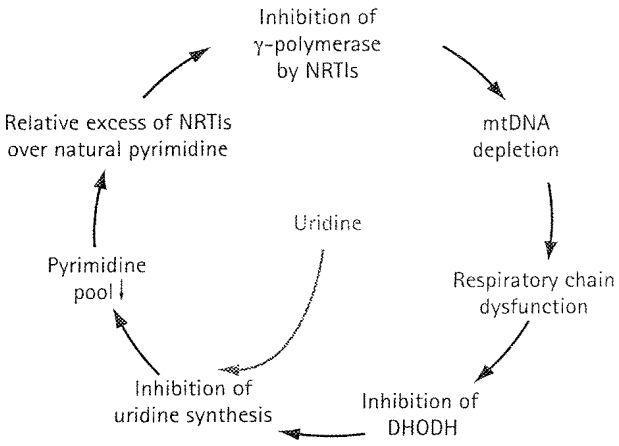
Figure 2. Molecular and functional consequences of diminished intracellular pyrimidine supply



UMP, uridine monophosphate.

Figure 3. Vicious cycle which is hypothesized to contribute to the mt toxicity of antiretroviral pyrimidine d-drugs and which may be abrogated by uridine supplementation



mt, mitochondrial; DHODH, dihydroorotate dehydrogenase; NRTI, nucleoside reverse transcriptase inhibitor.

death and grow virtually normally if the intracellular pyrimidine pools are replenished by substances that can be salvaged into pyrimidines by being converted into UMP distal to DHODH. One such substance that can bypass the block in the *de novo* synthesis of pyrimidines is uridine [32].

Uridine abrogates mt toxicity *in vitro*

The relationship between respiratory chain dysfunction and pyrimidine metabolism makes uridine an attractive candidate to alleviate symptoms of NRTI-related mt toxicity. Early work has demonstrated that neuronal cells exposed to ddC are rescued from death and improve in proliferation and neurite outgrowth if the medium was supplemented with uridine (50 μM) [33]. Uridine in concentrations of 50 μM also completely reversed the haematopoietic toxicity of AZT (5 μM) on normal human granulocyte–macrophage progenitor cells [34]. Similar strategies in mouse models of AZT-induced bone marrow suppression reversed anaemia, leucopoenia, increased peripheral reticulocytes and increased bone marrow cellularity [35]. The mechanism for the beneficial action of uridine on AZT is still unclear. As discussed above, AZT has many effects on cell metabolism [8,19]. It is conceivable that uridine or its derived pyrimidines may compete with AZT for one or several of these metabolic steps or, alternatively, for kinases or transporters responsible for the intramitochondrial presence of triphosphorylated AZT [8,34].

Investigations into a model of d-drug-related hepatotoxicity made the surprising discovery that uridine was not only able to prevent cell death (an expected finding), but also to prevent the onset of a severe mtDNA depletion and thereby normalize the synthesis of mtDNA-encoded respiratory chain subunits. This also normalized the rate of lactate production and the intracellular triglyceride content [36]. Importantly, uridine was only able to improve the mtDNA depletion caused by pyrimidine NRTIs, not that caused by purine analogues such as ddI.

The ability of uridine to antagonize the polymerase γ inhibition by pyrimidine d-drugs may be explained by its ability to disrupt the following vicious circle (Figure 3): as discussed above, polymerase γ inhibition involves competition of NRTIs with the natural nucleotides as a first step. mtDNA depletion, respiratory dysfunction, DHODH inhibition and pyrimidine depletion ensue. The decrease in intracellular pyrimidines most probably allows for a more efficient competition of the exogenous nucleoside analogue at polymerase γ. Thus, a vicious circle is closed and drives the cell into further mtDNA depletion. We hypothesize that this circle is disrupted by supplying uridine as an exogenous source of intracellular pyrimidines. The data also suggest that the ability of uridine to abrogate mt toxicities was proportional to the concentration of uridine [33,34,36], underlining the hypothesis of a competitive process. Alternatively, uridine may compete with antiretrovirals at steps of intracellular NRTI transport and phosphorylation.

Most recently, long-term exposure of adipocytes to d4T (10 μM), ddC (0.2 μM) or AZT (1 μM) was shown to induce a lipoatrophic phenotype consisting of apoptosis, loss of lipids, mtDNA depletion, loss of mtDNA-encoded respiratory chain subunits and disruption of the mt membrane potential [37]. The addition of uridine (200 μM) completely abrogated all these effects on adipocytes.

Notably, uridine was not only able to prevent the onset of mt toxicity but also to treat toxicities that were already established [35,36]. Interestingly, in the absence of uridine it took considerably longer for mtDNA depletion to develop (weeks), than it took for uridine to revert such mt toxicity (days) [36]. This relatively quick therapeutic effect of uridine relative to the more prolonged development of mt toxicities may allow for intermittent uridine dosing in order to 'reset the mitochondrial clock'.

Metabolism, pharmacokinetics and safety of uridine in humans

Normal uridine concentrations range from 3–8 μM in human blood plasma, bone marrow and cerebrospinal fluid [38]. Although uridine is part of our everyday food, diet is not an important source of uridine [39,40]. Clinical studies and animal models suggest that uridine is mostly produced in the liver and that erythrocytes serve as carriers for distributing the uridine throughout the body [38]. Exogenous uridine rapidly disappears from plasma ($t_{1/2}$=2 min), reflecting a concentrative and, under physiological conditions, unsaturated entry into tissue cells, as well as catabolism by the liver [41]. Subsequently, the tissue uridine pools turn over with half-lives of 13 to 18 h [41]. The physiological range of uridine in the human plasma was shown not to completely satisfy the pyrimidine requirements of dividing cells, making some *de novo* synthesis necessary for optimal proliferation [42]. Circulating uridine may nevertheless be of physiological importance by allowing dividing cells to utilize their salvage pathway [42].

Uridine has several metabolic fates in the cell (Figure 1). Exogenous uridine is rapidly incorporated into nucleotides in nucleated cells [43]. Uridine can be converted to dTTP and dCTP, which are used to produce DNA. UTP is used for the synthesis of RNA. UTP can also be converted into CTP, which upon conjugation of lipids forms cellular membrane constituents such as CDP ethanolamine. In the form of UDP sugars, uridine may help in the production of glycogen and in protein glycosylation. Uridine is degraded into beta-alanine, which can enter the tricarboxilic acid cycle (See Figure 1 for abbreviations) [38].

Pharmacokinetic and safety data for uridine were collected in several human Phase 1 and 2 trials. Parental administration of uridine as a 1 h infusion (8 g/m^2) resulted in plasma levels in the millimolar range, far above those required to abrogate NRTI-related mt toxicity [44]. Half-life, volume of distribution (634 ml/kg) and total clearance (4.98 ml/kg/min) of uridine appear to be independent of dose, whereas C_{max} and AUC increase with dose in a linear fashion [44]. In subjects given uridine at doses of 2–12 g/m^2 as a single 1 h infusion, about a fourth of the administered dose was excreted in the urine [44]. Uracil, a uridine catabolite, accounted for 3% of the uridine dose [44]. These intravenous doses were tolerated without side effects. However, transient fever, lasting for 15 min, occurred with higher doses [44,45]. Intermittent infusion schedules with 3 g/m^2/h for 3 h, alternating with a 3-h treatment-free interval, resulted in plasma levels of 138–335 μM during the treatment-free period and were tolerable over the 72-h study period [45]. Parenteral uridine necessitates central venous administration due to the onset of phlebitis if given through a peripheral vein [45]. Uridine can also be administered orally and is generally tolerated without any side effects. However, excessive oral dosing (12 g/m^2) is limited by mild and reversible osmotic diarrhoea due to the relatively poor bioavailability of uridine (7%) [46]. Using other pyrimidine precursors, for example, triacetyluridine [34,47,48] or inhibitors of uridine catabolism or excretion may also be envisaged [35,49,50].

Human uridine serum levels can now be effectively increased with mitocnol, a sugar cane extract with a high content (17%) of nucleosides [51]. 24-hour pharmacokinetic data indicate that consuming 36 g of a powder that contains mitocnol increases human uridine serum levels from baseline values (5.6 μM) to mean uridine serum concentrations (C_{max}) of 152.0 μM [51]. Adverse events were not observed. It is recommended that three sachets of mitocnol are taken on three consecutive days per month, taking into account the relatively quick improvement of mt toxicity from *in vitro* studies.

In summary, the current data indicate that uridine concentrations that are protective *in vitro* can be safely achieved with oral and parenteral dosing. Oral uridine supplementation (150 mg/kg/d) is also recommended and has been safely used long-term in patients with hereditary orotic aciduria, an inborn error of pyrimidine *de novo* synthesis, in which uridine reverses megaloblastic anaemia and other symptoms [52].

Interaction of uridine with antiretroviral nucleotides

If uridine or its metabolites are able to compete with NRTIs at the level of polymerase γ, they may also do so at the level of HIV reverse transcriptase (RT). This poses a theoretical risk for the antiretroviral efficacy of nucleoside analogues. The efficiency of RT inhibition is dependent on the ratio between the normal deoxynucleoside triphosphates and the NRTI triphosphates at the enzyme. For example, mycophenolate mofetil, an inhibitor of purine synthesis, depletes intracellular deoxyguanosine triphosphate and decreases plasma HIV-1 RNA in patients treated with the guanosine

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analogue abacavir [53,54]. Uridine may thus theoretically have an opposite effect on RT by increasing the normal deoxypyrimidine triphosphates.

Such an effect of uridine on the antiretroviral activity of pyrimidine analogues was first analysed with regard to AZT [34]. Phenotypic HIV resistance assays demonstrated that uridine did not interfere with viral suppression [34]. Importantly, uridine did not impair the antiretroviral activity even in a 10000-fold molar excess, whereas the maximal therapeutic effects of uridine were already achieved with a 10-fold molar surplus. Investigations in mice also came to the same conclusion [35].

The potential interference of uridine with the antiretroviral activity of NRTIs was also extensively examined in phenotypic HIV resistance assays using nucleoside analogues alone and in combinations [55]. Both X-4 tropic and R-5 tropic HIV isolates were tested and three different detection systems including primary human peripheral blood mononuclear cells were used. Uridine was added in concentrations up to 615 μM. Additionally, in these investigations no effect of uridine on NRTI-mediated viral suppression was detected. Enhancement of the normal intracellular pyrimidine stores therefore does not seem to have a crucial effect on HIV replication.

Taken together, the data suggest that the interaction between uridine and NRTIs in the prevention of mt damage does not necessarily imply a reduced antiretroviral efficacy. Explanations for this selectivity include a separate regulation of mt and cytoplasmic dNTP pools, either at the level of mt transport [56] or by the presence of disparate kinases in both compartments [57]. The differential action of uridine on the mt and antiretroviral replication enzymes may also be caused by differences between the polymerases in selecting the natural nucleotide over the activated NRTI.

Uridine in HIV-infected patients

The selective effect of exogenous uridine on NRTI-inhibited mtDNA replication, but not on NRTI antiretroviral action, implies that HIV-infected patients under treatment with pyrimidine NRTIs and suffering from mt toxicity may benefit from strategies aimed at increasing uridine. Mitocnol was used in an HIV patient with progressive hyperlactataemia, mt steatohepatitis and symptomatic elevation of creatine kinase (CK) under long-term antiretroviral treatment with d4T [58]. The patient was started on mitocnol (three sachets/day for four consecutive days). After 2 weeks, at his next visit, liver and muscle enzymes, as well as the myalgias had improved rapidly, despite unchanged medication. Lactate had normalized after 7 weeks and HIV replication remained below the limit of detection. d4T was then switched to tenofovir with no subsequent clinical or laboratory abnormalities.

Mitocnol is now widely used in Germany. Several and in-part randomized and placebo-controlled clinical trials are currently being conducted to formally analyse whether mitocnol is able to prevent and treat mt toxicities such as lipoatrophy, polyneuropathy, hepatic steatosis and myopathy. Virological failure has not been reported (UA Walker, personal communication).

Perspective

The issues discussed above have several further implications. Uridine supplementation may be used to enhance the therapeutic index of pyrimidine NRTIs and thus allow higher dosing to overcome multidrug resistance in salvage therapy. The available data also suggest the possibility of mtDNA depletion in blood [17,59,60]. If the detected mtDNA depletion in blood secondary to pyrimidine NRTIs indeed also reflects reduced mtDNA copy numbers in lymphocytes and if it exceeded a certain threshold, it would have effects similar to those of the direct DHODH inhibitor leflunomide and of inherited defects in pyrimidine synthesis. From the clinical experience with leflunomide as a licensed immunosuppressive antirheumatic drug, it could then be predicted that the mt toxicity in lymphocytes impairs the proliferation of lymphocytes in response to mitotic stimuli, interferes with CD4 recovery and thus is immunosuppressive. Impaired cell-mediated immune responses and reduced CD4 and CD8 lymphocyte subsets were also observed in several patients with an inherited defect in the *de novo* synthesis of pyrimidines; their immunodeficiency improved upon uridine therapy [52,61]. It was also shown that uridine antagonized the inhibition of lymphocytes by leflunomide [62,63]. Most recent *in vitro* and *in vivo* observations in HIV patients also support the view of mt toxicity as being immunosuppressive [11,64,65]. This also offers the potential for uridine to enhance the CD4 cell recovery of patients under antiretroviral treatment.

Strategies aimed at increasing uridine also improved symptoms in patients harbouring a qualitative defect in mtDNA by carrying inherited mtDNA mutations [66]. In patients with such mtDNA mutations, however, uridine would be predicted to ameliorate only one aspect of respiratory chain dysfunction, namely the consequences of DHODH inhibition and, in contrast with antiretroviral-treated HIV patients, not to improve the underlying mtDNA pathology. Therefore, patients with mtDNA mutations are likely to have a continued defect in ATP synthesis and hyperlactataemia under uridine. Further clinical data on this group of patients are eagerly awaited.

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Many aspects of uridine are still poorly understood. For example, an oral dose of 300 mg three times daily for 6 months also improved diabetic neuropathy in a well-conducted trial [67]. Therapeutic uses of uridine were also proposed in cardiovascular disease, hypertension, liver disease and infertility, among others [38].

Until the data from formal clinical studies are available, the risk–benefit calculation of using uridine in HIV-infected patients should be individualized. The current safety data justify the current use of uridine in individuals suffering from mt toxicity who are closely monitored and who cannot be switched to an antiretroviral regimen with a lower potential of mt toxicity.

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(54) Title: METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

(57) Abstract: The invention relates to methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity, that is, utilizing a bioavailable pyrimidine compound to ameloriate the toxic effects caused by leflunomide compounds.

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METHODS AND COMPOSITIONS FOR REDUCING TOXICITY ASSOCIATED WITH LEFLUNOMIDE TREATMENT

Leflunomide is an isoxazole derivative which has shown therapeutic potential in a diverse array of disease processes and conditions, e.g., as an antiinflammatory agent, an immunosuppressive agent, an anticancer agent and an antiviral agent.

Leflunomide is currently approved in the United States for use in the treatment of rheumatoid arthritis to reduce joint inflammation. It is marketed under the trademark ARAVA®.

U.S. Patent Nos. 5,624,946 and 5,688,824, incorporated herein by reference in their entirety, report that leflunomide has been used experimentally as an immunosuppressive agent in the treatment and prevention of chronic rejection in xenograft and allograft transplant recipients, both alone and in combination with other immunosuppressive agents.

In addition to data suggesting its value in treating, preventing and reversing acute and chronic rejection, U.S. Patent Application U.S. 2003/0114597, incorporated herein by reference in its entirety, reports that leflunomide has been shown to inhibit viruses of the Herpesviridae family *in vitro*.

U.S. Patent No. 4,965,276 describes the use of leflunomide to treat chronic graft versus host disease and other autoimmune diseases such as systemic lupus erythematosus (SLE). Leflunomide has also been shown to exhibit antineoplastic activity against certain tumors (Xu X et al., *Biochem. Pharmacol.* 1999; 58:1405) and may act by inhibiting tumor neoangiogenesis (Waldman WJ et al., *Transplantation* 2001; 72:1578).

Despite the reported therapeutic benefits of leflunomide in the treatment and prevention of these disease processes, it has also been noted that administration of leflunomide may produce dose-limiting toxicity. Toxicity associated with high doses of leflunomide include anemia, diarrhea, and pathological changes of the small intestine and liver. In a study of the anti-cancer effects of leflunomide (inhibition of the oncogene product PDGF and PDGFr) observable beneficial effects were reported but the doses required for these effects produced unacceptable incidence of side effects, including severe weight loss, anorexia and anemia. (Ko, Yoo-Joung, et al. *Clinical Cancer Research*, 2001;7: 800-805)

Recently, it has been suggested that uridine therapy reduces the toxicity of leflunomide without significantly impairing the control of allograft rejection afforded by leflunomide. The utilization of exogenous uridine occurs through the pyrimidine salvage

pathway in the face of the leflunomide's blockade of the *de novo* pathway. Notwithstanding the potential benefits of administration of exogenous uridine, therapeutic use of uridine is complicated by its poor bioavailability (about 8% - 10%), requiring high dose administration for effective therapy. Moreover, high doses of uridine may cause gastrointestinal complications, including diarrhea, which are poorly tolerated in transplant patients dependent on intestinal function for therapeutic drug administration and which may exacerbate the diarrhea already caused by the leflunomide.

The present invention relates to the surprising discovery that the use of orotic acid alleviates the toxicity typically observed with leflunomide administration. Orotic acid (also known as vitamin B_{13}), an intermediate in the uridine synthetic pathway, appears to eliminate the pyrimidine deficiency caused by the malononitrilamides, metabolites (analogues of the active metabolite) of leflunomide, while avoiding the problems associated with uridine administration.

Accordingly, the invention provides methods and compositions useful in alleviating or reducing toxicity associated with leflunomide administration without reducing its bioactivity, e.g., without reducing its immunosuppressive activity. The present invention uses a bioavailable pyrimidine compound to ameloriate the toxic effects (e.g., anemia, diarrhea, heptotoxicity) caused by leflunomide compounds. As a result, high doses of leflunomide compounds can be administered with minimal danger of toxicity, all the while maintaining the therapeutic efficacy of the leflunomide compound. Co-administration of a leflunomide compound with orally bioavailable pyrimidines, such as orotic acid, provides for treatment opportunities using leflunomide compounds previously believed to be toxic, e.g., the present invention provides methods of reducing the toxicity of A77 1726 (a metabolite of leflunomide) analogs (described hereinbelow) by co-administering a leflunomide compound and, e.g., orotic acid. In addition to orotic acid, it is contemplated that additional analogs and metabolites of orotic acid or other bioavailable pyrimidine compounds may be suitable.

In one aspect, the invention provides pharmaceutical compositions particularly for oral administration. Such pharmaceutical compositions suitably include a leflunomide compound, a bioavailable, especially an orally bioavailable, pyrimidine compound or a salt thereof, and a pharmaceutically acceptable carrier.

In another aspect, the invention provides a method of extending the dosage range of a leflunomide compound. The method involves co-administering to a subject, e.g., a mammal, an effective dose of a leflunomide compound and an orally bioavailable

pyrimidine compound or salt thereof, e.g., orotic acid. Thus, the invention provides a method of administering high doses of a leflunomide compound without developing, i.e., reducing, toxicity resulting from leflunomide administration, which method comprises administering to a mammal, e.g., a human, treated with a leflunomide compound an effective amount of a bioavailable pyrimidine compound.

The invention further provides methods of prevention or treatment of certain disease states or processes that are suitably treated with a leflunomide compound. Such disease states or conditions include transplant rejection.

The invention will now be described in detail, those skilled in the art will appreciate that such a description of the invention is meant to be exemplary only and should not be viewed as limitative of the full scope thereof.

The following definitions used in the art may be useful in aiding the skilled practitioner in understanding the invention.

"Ameliorating" means observably reducing, alleviating, inhibiting or diminishing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. For example, "amelioration of the effects of pyrimidine biosynthesis inhibition" may refer to any observable reduction in side effects caused by pyrimidine biosynthesis inhibition. Suitably, at least a 50% reduction in symptoms or side effects may be observed.

The term "co-administration" includes administration of two or more agents in a single unitary dosage form, administration of agents concurrently, and administration of agents sequentially, as long as they are given in a manner sufficient to allow both agents to achieve effective concentrations in the body. The agents may be in an admixture, as, for example, in a single tablet, or simply given concurrently. The agents may also be administered by different routes, e.g., one agent may be administered intravenously while the second agent is administered orally. In sequential administration, one agent may directly follow administration of the other or the agents may be administered episodically, i.e., one can be given at one time followed by the other at a later time.

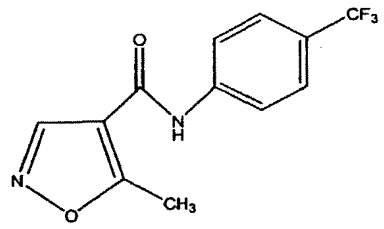
An "effective amount" of a compound, as used herein, means that amount of the compound or composition administered to a subject which is effective to produce its intended function, e.g., in one embodiment of the invention, prevention of transplant rejection. Thus, a "therapeutically effective amount" is an amount effective to produce therapeutic results. A "toxicity-reducing effective amount" is an amount effective to reduce toxicity. Typically, administration of effective amounts to a subject results in

observable amelioration of undesirable effects or symptoms of the condition or disease process which the subject is being treated.

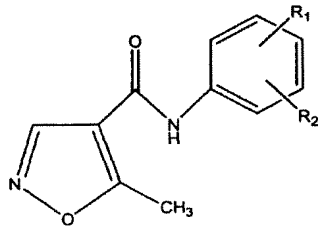
"Extending a dosage range" refers to providing a means by which greater doses of an agent may be administered to a subject to increase therapeutic effectiveness. Typically, extending a dosage range is useful, e.g., when efficacy of an agent is dose dependent but increased doses of the agent also leads to dose dependent toxicity. Alternatively, the term "extending a dosage range" may refer to administering agents believed to be toxic at any dosage.

A "leflunomide compound" refers generally to leflunomide, its analogs, its metabolites and analogs thereof.

Leflunomide is an isoxazole derivative with a chemical name of N-(4-trifluoromethylphenyl)-5-methylisoxazol-4-carboxamide having the following chemical formula (I):



Analogs of leflunomide which may be useful in the practice of the methods of the invention may be represented by formula (II):



(I) (II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and NH-CO-CH_2Br. (See, e.g., U.S. Patent Nos. 4,087,535; 6,133,301; and 6,727,272)

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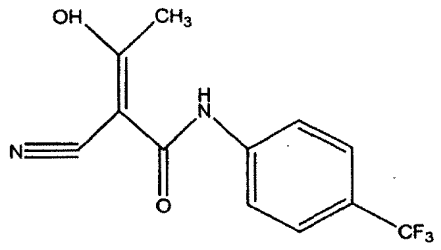
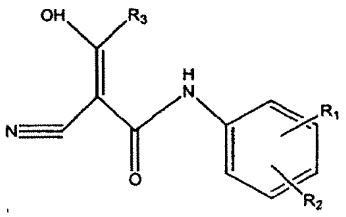
Leflunomide's active metabolite is referred to as "A77 1726" (2 cyano-3hydroxy-N-(4-trifluromethylphenyl)-buteneamide). After administration, leflunomide is rapidly converted to its active open-ring form, A77 1726, and is shown herein as formula (III):



(III)

This compound, a member of the malononitrilamide class of compounds, appears to account for leflunomide's activity and toxicity. Although its mechanism of action is not completely understood and wishing not be bound of any particular theory, A77 1726 is believed to exhibit at least two biochemical activities *in vivo*: inhibition of dihydroorotic acid dehydrogenase (DHODH) in the *de novo* synthesis of pyrimidine nucleotide triphosphates; and inhibition of selected tyrosine kinases involved in T-cell, B-cell, vascular smooth muscle cell, endothelial cell, fibroblast and tumor cell signaling cascades. A77 1726 also has been reported to block NFkB and AP-1 activation in peripheral blood lymphocytes *in vitro*. Additional mechanisms remain to be discovered.

Suitable malononitrilamide compounds which are analogs of A77 1726 may be useful in the practice of the methods of the invention and may be represented by formula (IV):



(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and NH-CO-CH_2Br and wherein R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl. Compounds of formula (IV) include FK7778 and FK779 wherein R_1 is -H, R_2

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is $-CF_3$ and R_3 is butynyl (i.e., 2-cyano- 3-hydroxy-N-[4-(lufluaromethyl) phenyl]-2-hepten-6-ynoic acid amide) and R_1 is -H, R_2 is cyano and R_3 is cyclopropyl (i.e., 2-cyano-3-hydroxy-3-cyclopropyl-N-(4-cyanophenyl)-propenic acid amide), respectively.

In some embodiments of the methods of the invention, the leflunomide compound is administered as a prodrug to subjects and subsequently converted *in vivo* to its active malononitrilamide compound, defined above. It is contemplated, however, that the malononitrilamide compound may also be directly administered, and the term "leflunomide compound", as defined above, also refers to malononitrilamide compounds. It is to be understood that discussion herein regarding leflunomide compound administration is meant to be inclusive of malononitrilamide compound administration, as appropriate.

Leflunomide and its analogs can be prepared by known methods such as those described in U.S. Patent No. 6,723,855; U.S. Patent No. 6,727,272; U.S. Patent No. 6,133,301; U.S. Patent No. 5,905,090; U.S. Patent No. 4,087,535; U.S. Patent No. 4,351,841; and U.S. Patent No. 4,965,276, all of which are incorporated herein by reference in their entireties. Leflunomide is also commercially available from chemical suppliers, such as SynQuest Corp. (Chicago, Illinois).

As used herein, "bioavailable" in reference to a pyrimidine compound is one that is at least about 20% bioavailable after administration. "Orally bioavailable" in reference to a pyrimidine compound is a compound that is at least about 20% bioavailable after oral administration.

The phrase "pharmaceutically acceptable carrier," as used herein, means a pharmaceutically-acceptable material, composition or vehicle, such as a liquid or solid filler, diluent, excipient, solvent or encapsulating material, involved in carrying or transporting the subject chemical from one organ, or portion of the body, to another organ, or portion of the body. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the subject. Some examples of materials which can serve as pharmaceutically-acceptable carriers include: (1) sugars, such as lactose, glucose and sucrose; (2) starches, such as corn starch and potato starch; (3) cellulose, and its derivatives, such as sodium carboxymethyl cellulose, ethyl cellulose and cellulose acetate; (4) powdered tragacanth; (5) malt; (6) gelatin; (7) talc; (8) excipients, such as cocoa butter and suppository waxes; (9) oils, such as peanut oil, cottonseed oil, safflower oil, sesame oil, olive oil, corn oil and soybean oil; (10) glycols, such as propylene glycol; (11) polyols, such as glycerin, sorbitol, mannitol and

polyethylene glycol; (12) esters, such as ethyl oleate and ethyl laurate; (13) agar; (14) buffering agents, such as magnesium hydroxide and aluminum hydroxide; (15) alginic acid; (16) pyrogen-free water; (17) isotonic saline; (18) Ringer's solution; (19) ethyl alcohol; (20) phosphate buffer solutions; and (21) other non-toxic compatible substances employed in pharmaceutical formulations. Wetting agents, emulsifiers and lubricants, such as sodium lauryl sulfate and magnesium stearate, as well as coloring agents, release agents, coating agents, sweetening, flavoring and perfuming agents, preservatives and antioxidants can also be present in the compositions.

As used herein, "preventing," "reducing risk" or "reduced risk" as it applies to a particular condition or disease process, refers to observable results which tend to demonstrate that a particular treatment or treatment regimen has resulted in a significant decrease in incidence of the condition or disease process in a treated population, as compared to an untreated or control population. Suitably, risk is reduced, or a condition is prevented, if at least 50% of the treated population are not afflicted.

As used herein, a "pyrimidine compound" refers to a compound that is bioavailable, especially orally bioavailable, and useful either directly or as intermediates in pathways for supplying pyrimidine nucleotides. A suitable pyrimidine compound is, e.g., orotic acid. Other suitable pyrimidine compounds include orotic acid salts, triacetyl uridine and salts thereof, cytidine, acylated cytidine and salts thereof.

It is to be understood that the phrase "a salt thereof," when used herein to refer to pharmaceutical compositions, means physiologically compatible salts which are pharmaceutically acceptable. Examples of suitable salts are alkali metal (e.g., sodium), alkaline earth metal (e.g., calcium, magnesium) and ammonium salts, including those of physiologically tolerated organic ammonium bases.

As used herein, the term "treating" means observably reducing any undesirable effect or symptom of a condition or process associated with a disease state or any undesirable effect of a treatment of a disease state. Suitably, at least a 50% reduction in symptoms or side effects may be observed in a treated subject.

It also is specifically understood that any numerical value recited herein includes all values from the lower value to the upper value, i.e., all possible combinations of numerical values between the lowest value and the highest value enumerated are to be considered to be expressly stated in this application. For example, if a concentration range is stated as 1% to 50%, it is intended that values such as 2% to 40%, 10% to 30%, or 1%

to 3%, etc., are expressly enumerated in this specification. These are only examples of what is specifically intended.

In one embodiment, the present invention provides an effective method for reducing the risk of toxicity of leflunomide compounds used for the treatment of transplant rejection. Particularly, the present invention relates to therapeutic methods for ameliorating the risk of toxic side effects of a leflunomide compound, and thus permitting extending dosing of such compounds. The present invention provides treatment of a patient suffering from the toxic side effects of a leflunomide compound with an orally bioavailable pyrimidine compound based on a novel treatment protocol. The pyrimidine compound is suitably orotic acid, a salt thereof (e.g., sodium orotate), or a triacetyluridine. The pyrimidine compound is provided to the patient to significantly reduce the toxic effects of a leflunomide compound, e.g., anemia and diarrhea resulting in reduced hematocrit and weight loss. These attributes are achieved through specific properties of the pyrimidine compounds and the novel treatment protocol as described herein.

A suitable pyrimidine compound is orotic acid. Orotic acid is found in small concentrations in the blood of healthy individuals. Elevated levels appear to be free of any appreciable complications in humans and animals. Several conditions are known, however, in which orotic acid levels in the blood are elevated, e.g., in urea synthesis defects, in individual Hereditary Oroticaciduria treated with uridine for years, and in patients receiving allopurinol, without recognized specific damage. In addition, it is recognized that blood levels of orotic acid are elevated several fold in renal failure without specifically recognized toxicity.

Orotic acid may be prepared by condensation of urea with the monoethyl ester of oxalacetic acid in methanol. Other preparation methods, including those utilizing biotechnological methods known in the art, are also suitable. Orotic acid may be administered in its free acid form, or may be administered as a pharmaceutically acceptable salt. Examples of suitable salts are alkali metal (e.g., sodium orotate), alkaline earth metal (e.g., magnesium orotate or calcium orotate) and ammonium salts, including those of physiologically tolerated organic ammonium bases. Orotic acid is also commercially available from chemical suppliers, such as Aldrich (Milwaukee, Wisconsin).

Also included among the bioavailable pyrimidine compounds of the invention are those comprising certain known acyl derivatives of uridine, i.e., acylated uridines, e.g., 2', 3', 5'-tri-O-acetyl uridine (or triacetyluridine (TAU)), 2', 3', 5'-tri-O-propionyl uridine, or 2', 3', 5'-tri-O-butyryl uridine. TAU, for example, is orally bioavailable. TAU and other

acyl derivatives of uridine can be made by methods known in the art (see, e.g., U.S. Patent No. 6,316,426; U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference); TAU is also commercially available through SP-Chemicals, Ludwigshofen, DK.

The pyrimidine compounds of the invention also include cytidine and certain acyl derivatives of cytidine, i.e., acylated cytidines, e.g., 2', 3', 5'-tri-O-acetyl cytidine (or triacetylcytidine or TAC), 2', 3', 5'-tri-O-propionyl cytidine, or 2', 3', 5'-tri-C-butyryl cytidine. TAC and other acyl derivatives of cytidine can be made by methods known in the art (see, e.g., U.S. Published Patent Application 2002/0035086 and references cited therein, all of which are incorporated herein by reference).

Suitably, the pyrimidine compound may be administered in an amount that is approximately that which is needed to provide the daily pyrimidine synthesis requirements minus what is provided through the salvage pathway. The total pyrimidine synthesis in adult humans is estimated to be from about 4 mmol/day to about 12 mmol/day, or about 450 to about 700 mg of uridine per day. (Bono VH, Weissman SM, Frei E. The effect of 6-azauridine administration on de novo pyrimidine production in chronic myelogenous leukemia. *J Clin Invest* 1964; 43:1486; Smith LH Jr. Pyrimidine Metabolism in Man. *New Engl J of Med* 1973; 288:764-772.) For orotic acid, this would amount to approximately 1000 mg per day. It is not believed to be necessary, however, to provide the entire daily supply of pyrimidine since the salvage pathway provides some of the total. It is believed that the bioavailability of orotic acid is approximately 50%. Therefore, for oral administration in an adult, an effective amount of orotic acid would be about 500 mg to about 2,000 mg per day. A similar dosing is contemplated for TAU.

For patients being treated with a leflunomide compound, the targeted blood level of active metabolite (A77 1726) is suitably between about 50 μg/mL and about 100 μg/mL. The maintenance dose may be adjusted by one of ordinary skill in the art to attain the desired blood level range of active metabolite. If pyrimidine deficiency is prevented with co-administration of a pyrimidine compound, the targeted blood level of active metabolite may be substantially higher, e.g. about 200 μg/mL or about 600 μM.

Mammalian transplant recipients, such as kidney recipients and bone marrow recipients, may be suitably treated in accordance with the present invention. Typically, a human transplant recipient is administered a leflunomide compound at a dose of about 100 mg per day for five days, and then 40 mg per day thereafter as a maintenance dose. The co-administration of the leflunomide compound and a pyrimidine compound will extend

the therapeutic dose of the leflunomide compound to more than 200 mg/patient/day. This method will prevent the development of or reduce the risk of toxicity (e.g., anemia, diarrhea, hepatotoxicity) and will result in achieving concentrations of the leflunomide compounds that can suppress rejection. It is expected that the use of this methodology will allow up to 10-fold or higher increase in dosage level of leflunomide compounds with minimal danger of developing toxicity to the patient. In other words, the present invention provides a method of administering a toxic dose of a leflunomide compound by administering an effective amount of a pyrimidine compound. By "toxic dose" or "high dose" is meant a dose of the leflunomide compound which when administered to a mammal such as a human often results in the toxic effects, e.g., anemic and diarrhea as well as other pathological changes. In humans, a high dose may be more than 200 mg per day.

In administration of leflunomide compounds, toxicity-reducing effective amounts of the bioavailable pyrimidine compounds are co-administered to subjects with allografts or xenografts, thereby ameloriating the toxic effects of the leflunomide compounds, i.e., weight gain is promoted and hematocrit maintained, with significantly less risk of toxicity than is observed after the same amount of leflunomide compound alone is administered. The risk of toxicity, associated with the administration of high doses of leflunomide compounds, is lowered by co-administering the leflunomide with a pyrimidine compound, especially an orally bioavailable pyrimidine compound. Thus, the combination therapy for use in accordance with the present invention provides an improved therapeutic index relative to leflunomide compounds alone given in conventional protocols. The treatment protocol in accordance with the present invention provides reduced risk of toxicity, (e.g., improved weight gain and hematocrit) i.e., little or no clinical symptoms or signs of toxicity.

The pyrimidine compounds of the present invention given in the illustrated dosing regimen, thus, overcome the toxicities of leflunomide compounds and can be considered beneficial agents for the control and treatment of toxicity associated with treatment with leflunomide compounds. In such combination therapy, the leflunomide compound may be co-administered with the pyrimidine compound concurrently, sequentially, or in a unitary formulation. For efficiency, ease of administration and patient compliance, the latter is especially suitable.

A pharmaceutical composition of a leflunomide compound and a bioavailable pyrimidine compound is suitably formulated in unit dosage form of about 500 mg to about

2000 mg of pyrimidine compound and about 20 mg to about 100 mg of leflunomide compound. Lower doses of pyrimidine compound may be adequate for children or individuals with reduced clearance of pyrimidines, such as individuals with reduced kidney function or other conditions that might reduce pyrimidine elimination.

The dosage form of compositions of the invention is not particularly limited, and any form suitable for oral administration may be used in accordance with standard formulation procedures known in the art. Examples of dosage forms suitable for oral administration include, but are not limited to, solid formulations and aqueous formulations. Solid formulations suitable for oral administration include capsules, tablets, powders or granules, and may include excipients such as lactose, glucose, sucrose or mannitol; a disintegrator such as starch or sodium alginate; a lubricant such as magnesium stearate or talc; a binder such as polyvinyl alcohol, hydroxypropylcellulose or gelatin; a surfactant such as fatty acid ester; and a plasticizer such as glycerine, and the like. Aqueous formulations suitable for oral administration include solutions, emulsions, syrups and suspensions. Such formulations may also include sugars such as sucrose, sorbitol or fructose; glycols such as polyethylene glycol or propylene glycol, oils such as sesame oil, olive oil or soybean oil, antiseptics such as p-hydroxybenzoate, and flavors such as strawberry and peppermint.

While, perhaps, less convenient than an oral formulation, it is also contemplated that the compositions may be formulated for rectal administration in accordance with standard formulations procedures known in the art. Examples of dosage forms suitable for rectal administration include solid suppositories, mucoadhesive suppositories, solutions, suspensions, retention enemas, gels, forms and ointments.

It is further contemplated that a dosage form of the compositions in accordance with the present invention may be formulated for immediate release, delayed release or controlled release. Many controlled release systems are known in the art (see e.g., U.S. Patent 5,529,991). Sustained, controlled or directed release compositions can be formulated, e.g., in liposomes, via laser originated openings or those wherein the active compound is protected with differentially degradable coatings, such as by microencapsulation, multiple coatings, etc.

For example, in diffusional systems, the release rate of drugs is affected by their rate of diffusion through a water-insoluble polymer. There are generally two types of diffusional systems, formulations in which a core of drug is surrounded by polymeric membrane; and matrix devices in which dissolved or dispersed drug is distributed

substantially uniformly and throughout an inert polymeric matrix. In actual practice, many systems that utilize diffusion can also rely to some extent on dissolution to determine the release rate.

Common materials used as the membrane barrier coat, alone or in combination, include but are not limited to, hardened gelatin, methyl and ethyl-cellulose, polyhydroxymethacrylare, polyvinylacetate, and various waxes.

In matrix systems, three major types of material are frequently used in the preparation of the matrix systems which include insoluble plastics, hydrophilic polymers, and fatty compounds. Plastic matrices which have been employed include methyl acrylate-methyl methacrylate, polyvinyl chloride and polyethylene. Hydrophilic polymers include methyl cellulose, hydroxypropylcellulose, hydroxpropyl-ethylcellulose, and its derivatives and sodium carboxy-methylcellulose. Fatty compounds include various waxes such as carnauba wax, and glyceryl tristearate. These matrix systems are prepared by methods well known to those skilled in the art. These methods of preparation generally comprise mixing the drug with the matrix material and compressing the mixture into a suitable pharmaceutical layer. With wax matrices, the drug is generally dispersed in molten wax, which is then congealed, granulated and compressed into cores.

The most common method of microencapsulation is coacervation, which involves addition of a hydrophilic substance to a colloidal dispersion. The hydrophilic substance, which operates as the coating material, is selected from a wide variety of natural and synthetic polymers including shellacs, waxes, starches, cellulose acetates, phthalate or butyrate, polyvinyl-pyrrolidone, and polyvinyl chloride. After the coating material dissolves, the drug inside the microencapsule is immediately available for dissolution and absorption. Drug release, therefore, can be controlled by adjusting the thickness and dissolution rate of the coat. For example, the thickness can be varied from less than one μm to 200 μm by changing the amount of coating material from about 3 to 30 percent by weight of the total weight. By employing different thicknesses, typically three of four, the active agent will be released at different, predetermined times to afford a delayed release effect.

Approaches to further reducing the dissolution rate include, for example, coating the drug with a slowly dissolving material, or incorporating the drug into a formulation with a slowly dissolving carrier. Thus, encapsulated dissolution systems are prepared either by coating particles or granules of drug with varying thickness or slowly soluble polymers or by microencapsulation.

While it is contemplated that a unitary oral formulation containing both a leflunomide compound and a bioavailable pyrimidine compound provides ease of administration and patient compliance, it is also understood that the compounds may be administered separately but packaged together, e.g., in a blister pack, with instructions for administration.

Although examples of suitable dosage ranges are provided, it will be appreciated that the specific dosages administered in any given case will be adjusted in accordance with the specific compounds being administered, the disease to be treated, the condition of the subject and other relevant medical factors that may modify the activity of leflunomide, the response of the subject or the amount of bioavailable pyrimidine compound needed, as is well known by those skilled in the art. For example, the specific dose for a particular patient depends on age, body weight, general state of health, diet, the timing and mode of administration, the rate of excretion, and medicaments used in combination and the severity of the particular disorder to which the therapy is applied. Dosages for a given patient can be determined using conventional considerations, e.g., by customary comparison of the differential activities of the subject compounds and of a known agent, such as by means of an appropriate conventional pharmacological protocol.

The following examples are provide to assist in a further understanding of the invention. The particular materials and conditions employed are intended to be further illustrative of the invention and are not limiting upon the reasonable scope thereof.

Example 1: Effect of orotic acid administration on efficacy of leflunomide in the treatment of acute rejection

Lewis Rats which received heart transplants from Brown-Norway rats were observed for graft survival and inflammation (scored on a 0-3 scale, with 0 being no inflammation). Treatments included 0, 5, 10 or 15 mg/kg of leflunomide in combination with 0 or 100 mg/kg orotic acid. The results are tabulated below.

Number animals	Dose leflunomide (mg/kg/day)	Dose orotic acid (mg/kg/day)	Graft survival (days)	Inflammation score(Mean) (0-3)
8	0	0	6.9	3.0
8	5	0	>30	2.0
5	5	100	>30	2.3
5	10	0	>30	1.7
5	10	100	>30	1.8
5	15	0	>30	1.5
5	15	100	>30	1.6

Administration of leflunomide reduced the intensity of the rejection reaction, as shown by the inflammation score, in a dose-related fashion. Orotic acid did not significantly affect the efficacy of leflunomide to reduce the intensity of the rejection reaction.

Example 2: Effect of orotic acid on leflunomide toxicity as measured by changes in body weight

As noted previously, the most observed symptoms of experimental leflunomide-induced toxicity are anemia and diarrhea resulting in weight loss or reduced weight gain. Lewis rats with either an allograft or xenograft weighing between 200 and 235 grams were divided into four treatment groups. Each group received 30 mg/kg/day of leflunomide, a high, toxic dose: Group I received leflunomide only; Group II received leflunomide plus 36 mg/kg/day of sodium orotate by gavage; Group III received leflunomide plus 100 mg/kg/day of orotic acid by gavage; and Group IV received 250 mg/kg/day of uridine by IP injection. Weight of each rat was measured at week 1 and week 4 post commencement of therapy. The results are tabulated below.

GROUP I (leflunomide only)

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30	234	246	13
L30	211	236	25
L30	241	237	-4
L 30	253	264	11
L 30	246	255	9
L 30	203	253	50
			Mean: 20.8

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GROUP II

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O36	270	266	-4
L30 + O36	244	235	-9
L30 + O36	250	218	-32
L30 + O36	205	267	62
L30 + O36	198	240	42
L30 + O36	211	262	51
			Mean: 18.3

GROUP III

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + O100	210	250	40
L30 + O100	208	265	57
L30 + O100	209	250	41
L30 + O100	207	271	66
L30 + O100	210	268	58
L30 + O100	197	258	61
			Mean: 53.7

GROUP IV

Treatment-dosage (mg/kg/day)	Weight at Week 1 Grams	Weight at Week 4 grams	Change in body weight
L30 + U250	207	231	24
L30 + U250	204	242	38
L30 + U250	208	230	22
L30 + U250	197	241	44
L30 + U250	204	272	68
L30 + U250	211	275	64
			Mean: 43.3

* L30 refers to administration of 30 mg/kg per day of leflunomide; O36 refers to 36 mg/kg per day of orotic acid; O100 refers to 100 mg/kg per day of orotic acid; and U250 refers to 250 mg/kg per day of uridine given IP.

The results showed that the use of a combination of leflunomide and orotic acid or a salt thereof significantly improved weight gain compared to use of leflunomide alone.

Example 3: Effect of orotic acid on leflunomide toxicity as measured by hematocrit

The experiment of Example 2 was repeated in Lewis rats and the hematocrit measured weekly for four weeks. The rats receiving treatment were divided into five groups wherein each group received 30/mg/kg/day leflunomide, a toxic, high dose. Group

I received leflunomide only. Group II received the leflunomide dose plus 36/mg/kg/day of sodium orotate; group III received the leflunomide dose plus 100mg/kg/day of sodium orotate; group IV received the leflunomide dose plus 88mg/kg/day of orotic acid; and group V received the leflunomide dose plus 250mg/kg/day of uridine given IP. A baseline hematocrit was measured, and hematocrits of each rat were measured at weeks 1-4 post commencement of therapy. The results are tabulated below.

GROUP I

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30	51	47	39	31	17
L30	57	52	46	33	20
L 30	54	55	54	37	30
L 30	55	49	48	33	24
L 30	52	50	46	37	22
L 30	53	53	44	41	30
					Mean: 23.8

GROUP II

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O36	53	50	48	39	20
L30 + O36	57	50	49	32	14
L30 + O36	53	51	39	27	10
L30 + O36	54	52	49	47	44
L30 + O36	51	53	40	36	35
L30 + O36	52	49	44	30	20
					Mean: 23.8

GROUP III

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + O100	53	48	43	41	32
L30 + O100	52	49	51	46	44
L30 + O100	51	46	44	42	45
L30 + O100	57	53	50	41	33
L30 + O100	51	49	46	34	22
L30 + O100	52	51	53	43	46
					Mean: 37

GROUP IV

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30 + U250	52	47	48	50	46
L30 + U250	56	50	51	51	49
L30 + U250	54	46	43	41	25
L30 + U250	51	51	48	38	24
L30 + U250	53	49	54	51	44
L30 + U250	54	54	49	43	38
					Mean: 37.7

GROUP V

Treatment-dosage (mg/kg/day)	Hct Week 0	Hct Week 1	Hct Week 2	Hct Week 3	Hct Week 4
L30+ OA88	56	50	53	52	50
L30+ OA88	54	51	54	52	54
L30+ OA88	52	49	44	38	32
					Mean: 45.3

The results demonstrated that use of the combination of leflunomide and orotic acid or sodium orotate, provided significantly higher hematocrits than in the use of leflunomide alone.

As used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the content clearly dictates otherwise. Thus, for example, reference to a composition containing "a pyrimidine compound" includes a mixture of two or more pyrimidine compounds. It should also be noted that the term "or" is generally employed in its sense including "and/or" unless the content clearly dictates otherwise.

All publications, patents and patent applications referenced in this specification are indicative of the level of ordinary skill in the art to which this invention pertains. All publications, patents and patent applications are herein expressly incorporated by reference to the same extent as if each individual publication or patent application was specifically and individually indicated by reference. In case of conflict between the present disclosure and the incorporated patents, publications and references, the present disclosure should control.

The invention has been described with reference to various specific embodiments and techniques. However, it should be understood that many variations and modifications may be made while remaining within the spirit and scope of the invention.

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We claim:

1. A pharmaceutical composition in unit dosage form for oral administration comprising an effective amount of a leflunomide compound; and an orally bioavailable pyrimidine compound, salts thereof or a combination thereof; together in a pharmaceutically acceptable carrier.

2. The composition of claim 1 wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

3. The composition of claim 1, wherein the unit dosage contains 500 mg to 2000 mg of pyrimidine compound.

4. The composition of claim 1, wherein the leflunomide compound is leflunomide, A771726 or FK778.

5. The composition of claim 1, wherein the composition is formulated for controlled release.

6. The composition of claim 1, wherein the composition is formulated for rectal administration.

7. A pharmaceutical composition comprising a formulation for oral administration, the formulation comprising a therapeutically effective amount of leflunomide, and orotic acid or a salt thereof, and a pharmaceutically acceptable carrier.

8. A method of reducing toxicity associated with administration of a leflunomide compound to a patient in need thereof, comprising administering to the patient a toxicity-reducing amount of a bioavailable pyrimidine compound.

9. The method of claim 8, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyaluridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

10. The method of claim 8, wherein the pyrimidine compound is administered orally.

11. The method of claim 8, wherein the pyrimidine compound is administered in a daily dosage of from about 500 mg to about 2000 mg.

12. The method of claim 8, wherein the pyrimidine compound is co-administered substantially simultaneously with the leflunomide compound.

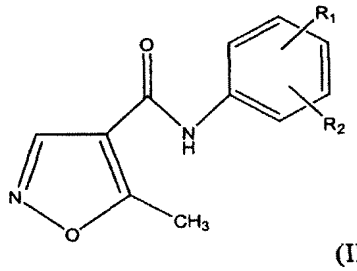
13. The method of claim 8, wherein the patient is a recipient of a transplant.

14. The method of claim 13, wherein the transplant is an allograft or a xenograft.

15. The method of claim 13, wherein the transplant is a heart, a kidney or bone marrow.

16. The method of claim 8, wherein the leflunomide compound is selected from a compound having

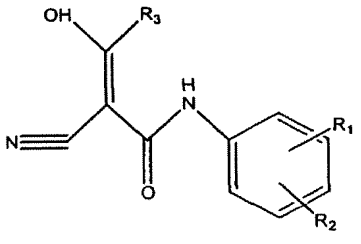
a) formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and -NH-CO-CH_2Br;

or formula (IV):



(IV)

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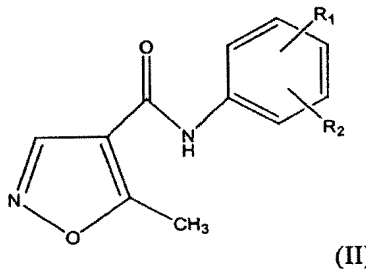
wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and -NH-CO-CH_2Br, and R3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl.

17. A method of extending the dosage range of a leflunomide compound comprising co-administering to a subject:

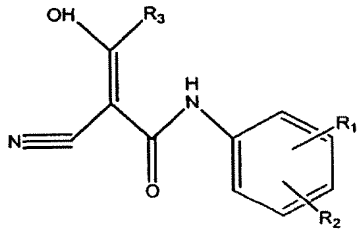
a) an effective amount of a leflunomide compound of formula (II):



(II)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, - -NH-CO-CH_2Cl and -NH-CO-CH_2Br;

or formula (IV):



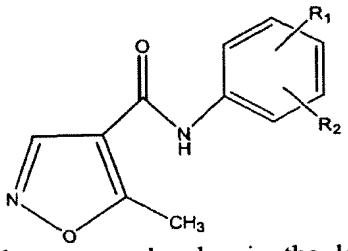
(IV)

wherein R_1 and R_2 are independently selected from the group consisting of $-CF_3$, –H, -Cl, -F, -Br, -CN, -COOH, $-OCH_3$, -NH-CO-CH_2Cl and -NH-CO-CH_2Br; and R_3 is selected from the group consisting of C_{1-5} alkyl, C_{2-5} alkenyl, C_{2-5} alkynyl, and C_{3-6} cycloalkyl; and

b) a toxicity-reducing amount of an orally bioavailable pyrimidine compound selected from the group consisting of orotic acid, a salt thereof, triacetyl uridine, a salt thereo, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof.

18. A method of administering a toxic dose of a leflunomide compound to a mammal, comprising administering to the mammal an amount of an orally bioavailable pyrimidine compound sufficient to reduce the toxic effects of the leflunomide compound.

19. A method of reducing toxicity associated with the administration of a therapeutically effective amount of a leflunomide compound to a mammal, comprising: orally administering to the mammal a bioavailable pyrimidine compound selected from orotic acid, a salt thereof, triacetyluridine, , a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, and a combination thereof, in an amount effective to reduce the toxicity without blocking therapeutic

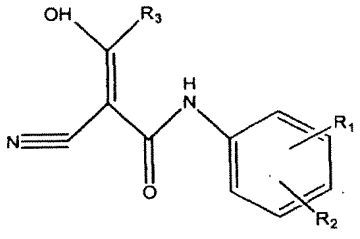


effect of the leflunomide compound, wherein the leflunomide compound is a compound of formula (II)

or formula (IV).

20. The method of claim 19 wherein the pyrimidine compound is orotic acid or a salt thereof.

21. A method of treating rejection in a transplant recipient comprising co-



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administering a therapeutically effective amount of a leflunomide compound and a toxicity-reducing effective amount of bio-available pyrimidine compound.

22. The method of claim 21, wherein the pyrimidine compound is orally bio-available.

23. The method of claim 22, wherein the pyrimidine compound is orotic acid, a salt thereof, triacetyl uridine, a salt thereof, cytidine, a salt thereof, an acylated cytidine, a salt thereof, or a combination thereof.

24. A method of achieving an effect in a patient comprising co-administering an effective amount of a leflunomide compound and an effective amount of orotic acid, a salt thereof, triacetyl uridine, a salt thereof, or a combination thereof, wherein the effect is treatment of rejection of a transplant, wherein the transplant is heart, kidney or bone marrow.

25. A pharmaceutical combination comprising a packaging having a plurality containers, at least one container containing a leflunomide compound, at least one other container containing a bioavailable pyrimidine compound, and an instructions for co-administering the leflunomide compound and the pyrimidine compound to a subject who is a transplant recipient.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US05/26145

A. CLASSIFICATION OF SUBJECT MATTER
IPC(7) : A61K 31/42
US CL : 514/378
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
U.S. : 514/378

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)
WEST

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 6,133,301 A (BARTLETT) 17 October 2000(17.10.2000), see entire document.	1-25

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:

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"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

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APPENDIX C

RELATED PROCEEDINGS

None